ABSTRACT OF THESIS

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Title of Thesis NUCLEIC ACID METABOLISM

The main part of this thesis is an electron microscope study of myogenesis in embryos of the toad Xenopus laevis. When anucleolar mutants of the animal are treated with tritiated uridine only non-ribosomal RNA becomes radioactively labelled and this is visualized by electron microscope autoradiography. In this way the cellular kinetics of non-ribosomal RNA synthesis and transport during myogenesis have been investigated.

Parallel investigations concerning DNA metabolism, the cellular distribution of the enzyme RNA-polymerase and the cellular distribution of poly-adenylic acid sequences, known to be attached to certain types of RNA, have also been performed.

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NUCLEIC ACID METABOLISM

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Ph.D.
University of Edinburgh

1973

CONTENTS

More detailed contents are given at the beginnings of each chapter.

CHAPTER ONE

GENERAL INTRODUCTION

Most of the work in this thesis is done on embryos of Xenopus laevis (a toad), homozygous for a deletion of the ribosomal cistrons and called 'anucleolar'. In 1958 Elsdale et al discovered some Xenopus which had only one nucleolus per nucleus instead of two. When mated a quarter of the offspring had two nucleoli, half had one and the remaining quarter had no nucleoli. The character was obeying the classical Mendelian laws; it was a single gene. The anucleolar embryos became microcephalic and oedematous and died at the swimming tadpole stage. Kahn in 1962 looked at the mitotic chromosomes of wild-type (2-nu) and the heterozygous (1-nu) embryos. In the wild-type he observed two large chromosomes each with a sub-terminal centromere and a secondary constriction in each of the short arms, near the centromere. In the 1-nu chromosomes only one such chromosome per metaphase plate was observed. The secondary constriction, presumed to be the nucleolar organizer, is deleted in the mutant chromosome. In 1964 Brown & Gurdon labelled the RNA of wild-type and anucleolar Xenopus embryos and extracted the RNA and characterized it. The wild-type embryos clearly showed synthesis of ribosomal

RNA whereas this type of RNA synthesis was lacking in 0-nu embryos. This became one of the important pieces of evidence for the identification of the nucleolus as the site of ribosomal RNA synthesis. Wallace & Birnstiel (1966) (also see Birnstiel et al 1965) extracted rRNA from Xenopus and hybridized it to saturation with DNA from 2-nu, 1-nu and 0-nu embryos. The amount of DNA complementary to rRNA was directly proportional to the number of nucleolar organizers present. The anucleolar embryos then do not possess any detectable ribosomal cistrons.

The important point in this work is that the animal synthesizes no detectable rRNA. This offers a unique opportunity to study the cellular metabolism of non-ribosomal RNA, including messenger RNA directly by autoradiography. Also, by comparing results between 0-nu and 2-nu animals it is hoped to learn something of the behaviour of the normal nucleolus and the transcriptional organization of the nucleus in general. Wallace (1966) undertook a similar study using light microscopy autoradiography. Ken Jones of this Institute (my supervisor) extended this to the electron microscope paying particular attention to the blobs (Wallace 1966) present in the 0-nu nucleus and to myofibrils. This work extends these preliminary findings.

By examining myofibrils it was hoped to learn something of the behaviour of specific messenger RNA species. In myofibrils the two major proteins, actin and myosin are spatially well segregated. The early results of Jones indicated that newly synthesized non-ribosomal RNA associates with myofibrils. It seemed reasonable to conclude that specific messenger RNAs, probably coding for actin and myosin, were associated with their respective protein products. This phenomenon is more fully analyzed in Chapter 3 of this thesis. In addition a surprising conclusion emerged from these results concerning the kinetics of tritiated uridine labelling of myofibrils; it appeared that much of this myofibril-associated non-ribosomal RNA was not nuclear in origin. It appeared in myofibrils well before nuclei lost any detectable newly synthesized RNA in a chase experiment.

The evidence for myofibrillar RNA being non-nuclear in origin was not absolutely conclusive. The quality of the results is fully discussed in Chapter 3.4. So it was only with caution that it was concluded that cytoplasmic transcription was occurring, particularly because of the weight of evidence in other systems that all eukaryotic non-mitochondrial RNA is nuclear in origin. However in other experiments of an

entirely different nature the direct conclusions again supported the hypothesis of cytoplasmic transcription. It was found that myofibrils incorporate tritiated thymidine, a DNA precursor (Chapter 4), that interfibrillar regions of the cytoplasm become labelled in a cytological assay for DNA-dependent RNA polymerase (Chapter 6), and in a different organism, the dystrophic mouse, it was discovered that a nucleic acid apparently resistant to RNAse coextracts with myosin, and hence presumably DNA or DNA-protein complex is tightly associated with myosin (Appendix 7.1). These results, although preliminary in nature, do provide evidence for the existence of DNA, the transcription enzyme and nascent RNA in muscle cytoplasm. This unifying aspect of the work is separately discussed in the General Discussion on Cytoplasmic Transcription in muscle tissue (Appendix 7.2).

The work is unpublished except for the presentation of Ken Jones' work and some of my early results at the International Congress of Muscular Dystrophy at Perth, Australia in November 1971, and the Sixth Symposium on Current Research in Muscular Dystrophy and Related diseases at University College, London in January 1972, (Jones & Bacon 1972).

CHAPTER TWO

ULTRASTRUCTURAL OBSERVATIONS ON MYOGENESIS IN ANUCLEOLAR AND WILD-TYPE XENOPUS EMBRYOS

2.1 INTRODUCTION

The work in this chapter consists of purely morphological observations but some experimental work is mentioned in the introduction because of its importance in explaining the rationale of the approach. The work presented has the aim of demonstrating that the lesion in the mutant does not seriously affect the process of myogenesis, thus validating its use in the experiments described in the next chapter. In

addition some novel observations on the process of myogenesis were made. Myogenesis has been extensively reviewed by Konigsberg (1965), Yaffe (1969), Hermann, Heywood & Marchok (1970) and Fischman (1970). Unless otherwise stated the discussion refers to these reviews. Most of the morphological observations described in these reviews have been made on fresh or tissue culture muscle from rat and chick embryos.

A: Filamentogenesis

Filamentogenesis is the polymerization of myosin and actin monomers, possibly in conjunction with other proteins, to form thick and thin filaments as visible in the electron microscope. Apart from myosin polysomes (see Chapter 3.1) the first step visible in EM sections in the synthesis of myofibrillar apparatus proteins, is the appearance of the thick or the thin filament. Individual actin and myosin molecules can be visualized by purification, spreading on EM grids and negative staining but in sections of cells they are not distinguishable from background proteins. The individual proteins must join together in a specific way eventually so as to form the characteristic thick and thin filaments of the correct dimensions to fit into the mature myofibril. Actin filaments identified by their diameter, often appear scattered through the cytoplasm

with varying degrees of orientation, sometimes in loosely woven 'skeins' continuous beneath the plasma membrane, or in shorter, loosely stacked bundles or even as free non-orientated filaments, e.g. in chick embryos (Allen & Pepe 1965, Dessouky & Hibbs 1965, Przybylski & Blumberg 1966, Fischman 1967), chick tissue culture (Shimada et al 1967, Larson et al 1970), amphibian embryos (Hay 1961, Kelly 1969), rat embryos (Bergman 1962, Heuson-Stiennon 1964, Andersson-Cedergren & Karlsson 1967) and rat tissue culture (Cedergren & Harary 1964). There is a single contrary report of actin filaments first appearing already stacked in myofibrils which previously contained only myosin filaments in chick tissue culture (Firket 1967). These actin filaments, whether free or loosely associated with each other sometimes have ribosomes apparently attached to them, in chick embryos (Przybylski & Blumberg 1966), rat embryos (Bergman 1962), and amphibians (Hay 1961, Andersson-Cedergren & Karlsson 1967). Filaments with attached ribosomes were looked for and not observed in chick tissue culture and embryos (Shimada et al 1967, Fischman 1967). These free thin filaments were more definitely identified as actin filaments by their appearance as double helices of spherical subunits in negatively stained homogenates of chick embryo cells (Allen & Pepe 1965) and their ability in the homogenates

to bind heavy meromyosin (Ishikawa et al 1969). Thus although actin is synthesized on polysomes, as is myosin, there are conflicting observations concerning whether or not the product polymerizes into filaments while still attached to the polysome either in vivo (above) or in vitro (Allen & Terrence 1968, Heywood & Rich, 1968). Similarly thick filaments are seen freely and individually scattered throughout the cytoplasm in chick tissue culture (Shimada et al 1967), chick embryos (Przybylski & Blumberg 1966, Fischman 1967), rat embryos (Bergman 1962, Heuson-Stiennon 1964), rat tissue-culture (Cedergren & Harary 1964), and amphibians (Hay 1961, Andersson-Cedergren & Karlsson 1967, Kelly 1969).

In some cases free myosin filaments are never seen. Instead myosin filaments are first seen in bundles together with actin filaments in chick embryos (Allen & Pepe 1965) and chick tissue-culture (Larson et al 1969). Thus the evidence is conflicting even in the same tissues.

Ribosomes, sometimes in the form of long, helical myosin synthesizing polysomes (Chapter 3.1) are sometimes seen in association with myosin filaments

in chick embryos (Przybylski & Blumberg 1966), chick tissue-culture (Larson et al 1970), rat embryos (Heuson-Stiennon 1964) and rat tissue-culture (Cedergren & Harary 1964). These were looked for and not seen in chick tissue-culture (Shimada et al 1967) and chick embryos (Fischman 1967). The apparent association of ribosomes with myofilaments may really occur in the cell and be functionally significant. However the observations of association could be artefactual, due to chance juxtaposition of free entities or to association during fixation.

Failure to observe this association in some cases is not necessarily evidence against the association being real. For instance it could be proposed that fixation can destroy this association in some cases, that the duration of association is short, or that the associated structures are randomly aligned, thus making observation difficult. The work discussed in the results section adds to the weight of evidence for ribosomes being associated with myofilaments. The significance of this is discussed in the discussion section.

B: Fibrillogenesis

Fibrillogenesis is the association of thick and thin filaments, together with other myofibrillar components, such as Z and M band material, to form striated myofibrils. The observations in chick embryos and tissue-culture (Allen & Pepe 1965 and Larson et al 1969) of the first appearance of myosin filaments already stacked alternately with actin filaments implies that at least polymerization, if not also synthesis of myosin filaments takes place in close association with actin filaments. This phenomenon could have a length regulation, or template function in the polymerization process, or could be passive in that there is no restriction on even myosin monomers binding to actin. If this association is necessary for fibrillogenesis it would require actin to be polymerized before myosin (they could be synthesized at any time and myosin monomers could be stored in solution until actin is polymerized). Actin filaments are frequently observed earlier in development than myosin filaments in chick embryos (Allen & Pepe 1965 and Przybylski & Blumberg 1966), chick tissue-culture (Shimada et al 1967), and amphibian embryos (Kelly 1969) although the reverse is found in axolotl embryos

(Hay 1961) and chick tissue-culture (Firket 1967). Other evidence already cited against this theory is the observation of free myosin filaments at all stages of myogenesis.

In the results section of this chapter it is shown that in Xenopus embryo somites myosin filaments first appear in the form of stacked fibrils. Thus filamentogenesis appears to be coupled with fibrillogenesis. The further finding that these myosin-containing fibrils are associated with polysomes containing newly synthesized RNA shows that myosin synthesis is coupled with these other two processes. The Z band has been ascribed roles in fibrillogenesis. Kelly (1969) has argued that in amphibian embryos Z band material is laid down in longitudinally orientated actin filaments in the cell periphery and then the myosin filaments, found free in the cell interior, fit themselves in between the actin filaments, the Z band then contracting to draw the filaments closely together. This is deduced from the observation that the Z band becomes more compact and the myofibrils become more tightly organized towards the edge of the cell. He envisages a slow wave of Z band contraction and simultaneous myosin filament accretion from the cell periphery towards the centre. In rat embryos (Bergman 1962), rat tissue culture (Cedergren & Harary 1964)

and amphibian embryos (Hay 1961) Z band material is claimed to extend laterally from myofibrils and actin filaments are seen attached to these extensions. This is envisaged as a method of lateral growth of myofibrils, the myosin filaments fitting in between the attached actin filaments. Firket (1967) claims that in chick tissue culture longitudinally homogeneous actin/myosin fibrils are crossed by Z bands some time after the fibrils are laid down, and then the myosin filaments dissolve away for a short distance either side of the Z band leaving behind the actin filaments in the I band. There are other cases (chick embryos: Allen & Pepe (1965), Fischman (1967); chick tissue culture: Larson et al (1970)), where myofibrils from one to several sarcomeres in length are observed in the complete absence of Z band material. These fibrils definitely contain both thick and thin filaments and in some cases have been shown to be hexagonally stacked in the absence of Z band material. These authors then disclaim any function of Z band material in the hexagonal stacking of myofilaments. The Z band material may, however, be involved in the formation of striations, i.e. the registering of the two types of filaments, in these cases. Allen & Pepe (1965) and Larson et al (1970) show the simultaneous appearance of Z bands and the

registering of the filaments. Observations described in the results section support the theory of involvement of Z band material in registration but not in stacking.

As has been pointed out there have been several observations of complete actin and myosin filaments existing free in the cytoplasm and this implies that myofibrils grow by accretion of individual complete filaments. However there are several examples of ribosomes being observed in association with or clustered around myofibrils, implying that actual synthesis of at least some myofibrillar protein takes place in situ on the myofibril. These include Dessouky & Hibbs (1965) and Przybylski & Blumberg (1966) in chick embryos; Bergman (1962) in rat embryos; Cedergren & Harary (1964) in rat tissue culture; Andersson-Cedergren & Karlsson (1967) in adult amphibians; Jones (1971) in Xenopus embryos and Larson et al (1970 & 1969) in human, mouse and chick muscle. In the case of Jones (1971) and Andersson-Cedergren & Karlsson (1967) the ribosomes were the helical polysomes thought to be myosin synthesizing polysomes (Chapter 3.1). Fischman (1970) found that myofibril organization can be apparently completely disrupted by treatment of the cells with trypsin (in embryonic chick heart cells), the hexagonal array, striation

and filament orientation apparently being completely lost. Incubation in trypsin free medium, even in the presence of cycloheximide, results in the reformation of fibrils with a hexagonal array, giving strong support to the idea of self-assembly of myofibrils without the necessity of protein synthesis for assembly. However as he claims that only 90% of trypsin treated cells lose their fibrillar organization it is not clear if the intact myofibrils in the cycloheximide treated cells were in fact dissociated by trypsin. Attempts to reconstitute organized myofibrils by mixing the constituents in vitro have failed to produce more than an amorphous precipitate (Pinset-Harstrom 1968). Autoradiographic data (Chapter 3.1) strongly suggests that protein synthesis does occur within myofibrils. In this study no free filaments were seen and evidence is presented for the coupling of synthesis with both filamentogenesis and fibrillogenesis.

2.2. MATERIALS AND METHODS

Embryos between the stages 11-18 (Rugh 1948) from the uridine labelling experiment (Chapter 3) were used. This means that they were fixed 15 hours after staging. The methods are fully described in Chapter 3.2. Instead of processing the sections for autoradiography they were examined directly on an AEI EM6G electron

microscope at 60kV, 50 micron objective aperture. Uncorrected magnifications are quoted. Some observations are, however, taken from autoradiograms and here, unless stated, the silver grains should be ignored.

2.3 RESULTS

A: Observations on Wild Type (1 & 2-nu)Embryos

All wild-type muscle cells at all stages contained nuclei with convoluted membranes, dispersed chromatin and compact nucleoli with fibrous and granular components. The cytoplasm always contained many large lipid droplets and yolk granules, glycogen, ribosomes, round or elongated mitochondria and smooth endoplasmic reticulum, shown in Plates 1, 2, 5 and 8.

Specially noted structures were:

(1) Mature myofibrils (plates 1 and 2). These contain thick and thin filaments (160Å and 40-70Å in diameter respectively as measured in Plate 3) and have clear A and I bands and M and Z lines. Sarcoplasmic reticulum and transverse tubules are present (Plates 2 and 3). Striations are roughly in phase whenever two or more striated myofibrils lay side by side (Plates 1 and 2). Plate (3) shows apparently imperfect hexagonal stacking of myofilaments, but this may be due to the section being slightly oblique.

(2) Minifibrils. These are clusters of up to 50 thick filaments about 7000Å to 16000Å long. They may be longer as the minifibrils are randomly orientated in the sections. The individual filaments are about 180Å in diameter and thus are probably myosin filaments (Plates 4 and 5). Thin actin filaments cannot always be seen in minifibrils. Minifibrils are studded with ribosomes which are not seen to be arranged into long helices characteristic of myosin synthesizing polysomes. Traces of minifibrils are occasionally seen to be partly embedded in polysome clusters (see below). Sometimes several minifibrils are associated together at one end forming a star-like arrangement (Plate 6). In this configuration long, helical polysomes are occasionally seen (arrows in Plate 6) lying alongside myofilaments. Plate 5 has a cross-section of a minifibril showing that stacking is not perfect. Minifibrils are not seen in the vicinity of mature myofibrils (Plates 1 and 2).

(3) Loose collections of minifibrils in parallel orientation, occupying a roughly cylindrical region of the sarcoplasm, often parallel to existing mature myofibrils (Plate 7). The minifibrils here contain thin as well as thick filaments (arrow in Plate 7).

(4) Polysome clusters. These are large aggregations of ribosomes in linear clusters embedded in a dense, amorphous substance. The ribosomes have a diameter of about 210Å (Plates 6, 8, 9, 10) as compared with a diameter of about 260Å as measured on the ribosomes of rough endoplasmic reticulum in other sections. In Plate 6 it can be seen that a long helical polysome is embedded in a cluster. The clusters are probably spherical with a diameter of about 18000Å as they appear circular in sections in a variety of planes as the plates show. Plate 8 shows that polysomes clusters and myofibrils advanced in development can exist in the same cell at the same time. Occasionally portions of minifibrils are seen partly embedded in a cluster (Plates 6 and 10). Plate 11 shows a polysome cluster lying alongisde a mature myofibril. Polysome clusters are larger than minifibrils.

(5) Free polysomes. When seen free in the sarcoplasm these large, myosin synthesizing polysomes have the most ordered arrangement of monosomes (Plate 12)$\frac{1}{2}$ The largest observed had 40-50 visible monosomes of about 200Å in diameter. Transverse sections of these polysomes are seen in Plate 15 (which is O-nu material), which shows again the helical arrangement of the polysome, having about

5 monosomes per turn, with a polysome diameter of 350-450Å.

(6) Small patches of amorphous, lightly staining material, perhaps associated with ribosomes, in areas containing mainly glycogen (Plate 13).

Structures not seen:

The subsarcolemmal skeins of thin filaments referred to in the introduction, seen in other material by other authors were not seen in Xenopus tissue despite a careful search in these regions. A typical subsarcolemmal region is shown in Plate 14. Individual actin and myosin molecules cannot of course be expected to be recognized in sectioned material. Actin polysomes have an expected size of 15-25 ribosomes (see Chapter 3.1) and thus may not be statistically distinguishable from random collections of free ribosomes or parts of myosin polysomes. Free actin and myosin filaments were not seen. They are only seen in fibrils and minifibrils. Amorphous lateral extensions of the Z line, seen in other amphibians as referred to in the introduction, were not observed here (e.g. plate 8 top right). Intermediate filaments (100Å diameter) reviewed by Fischman (1970) were not observed here.

PLATES (1) - (14) : WILD TYPE

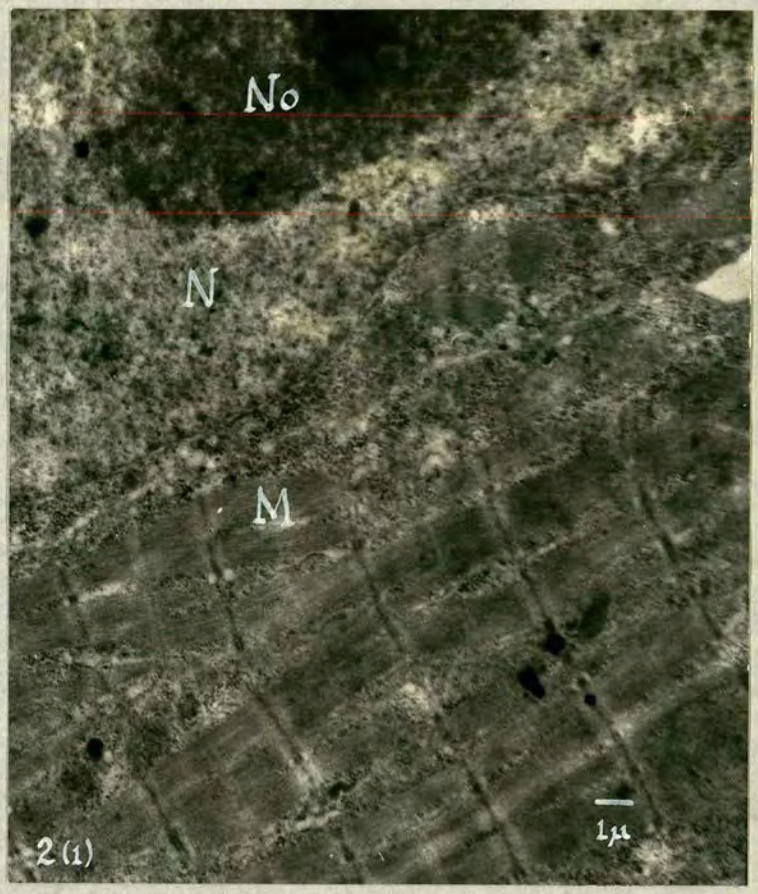


PLATE (1) Mature myofibrils (M), nucleus (N) and nucleolus (No). Stage 18 (gill-bud).

PLATE (2). Mature myofibrils, yolk platelets (Y), lipid droplet (L) and mitochondrion (Mc). Stage 18.

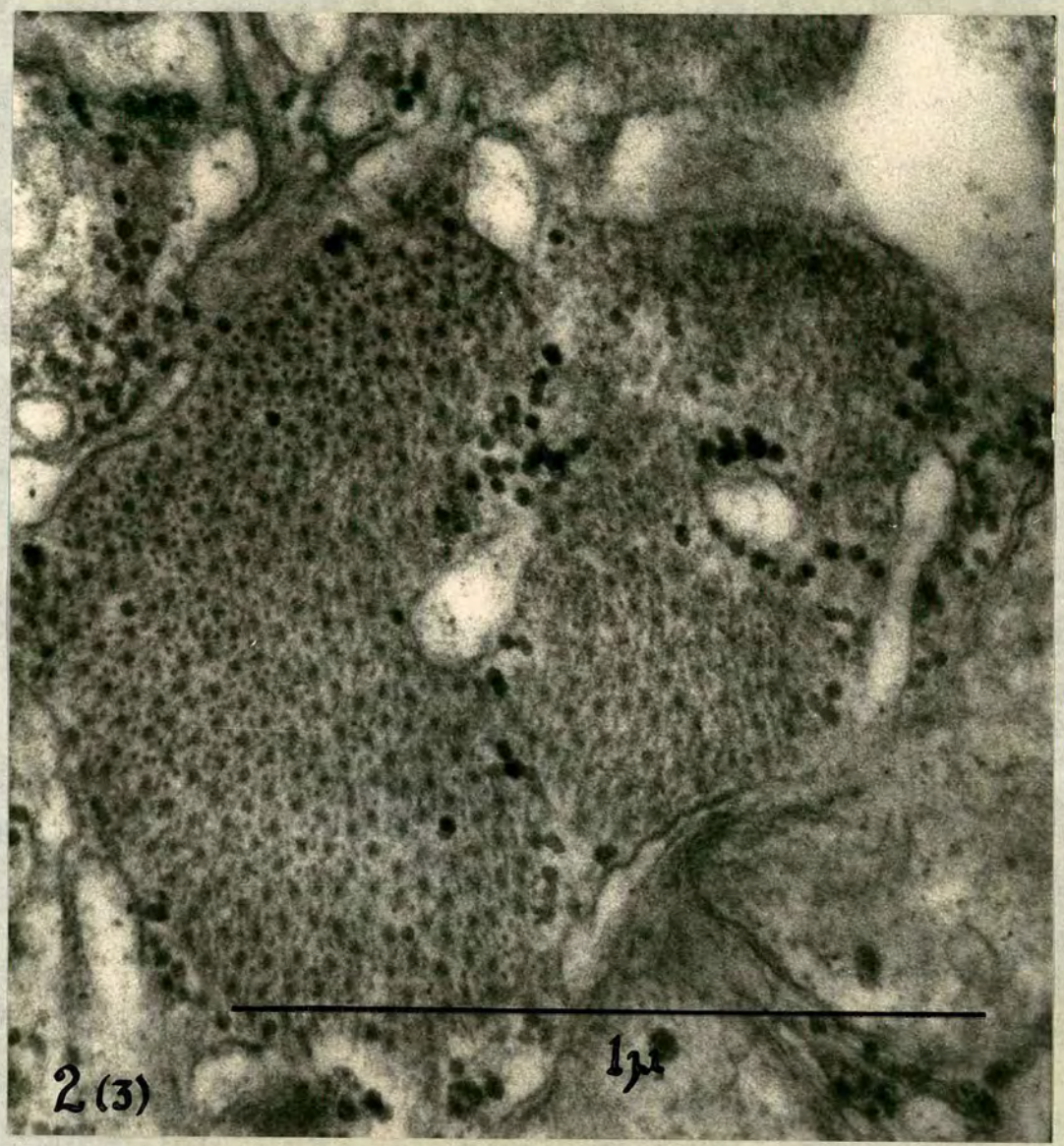


PLATE (3) Cross section of mature myofibril showing thick and thin myofilaments. Stage 17 (muscular response).

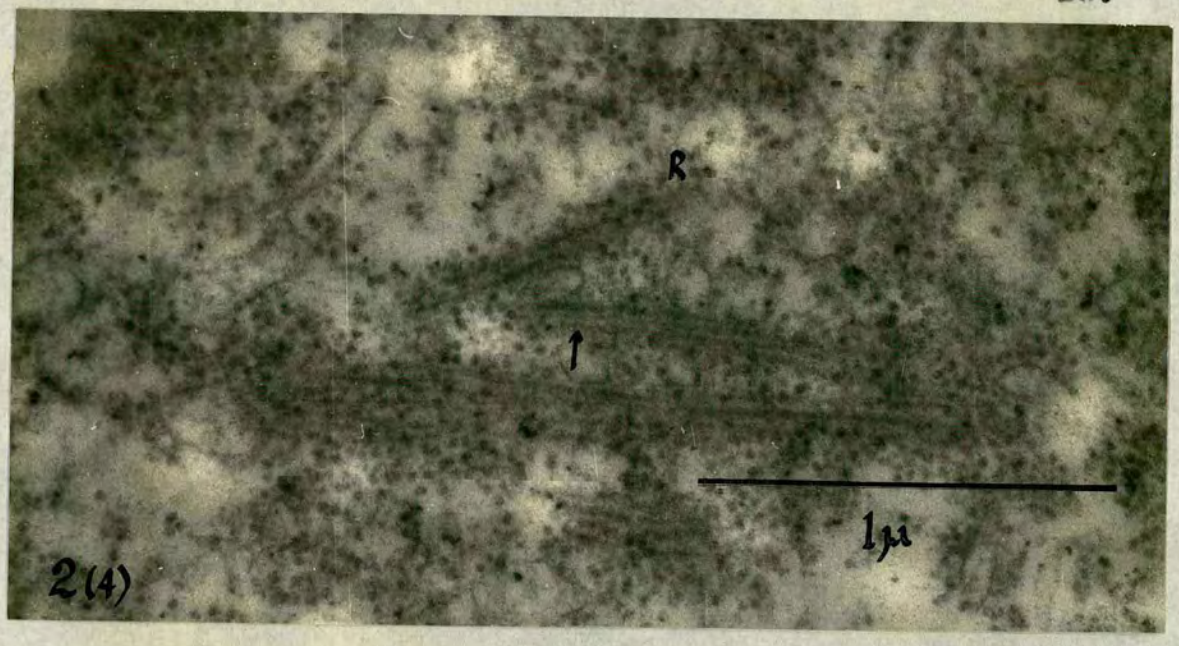


PLATE (4) Minifibrils showing both thick and thin myofilaments (arrow) and ribosomes (R). Stage 11 (late gastrula).

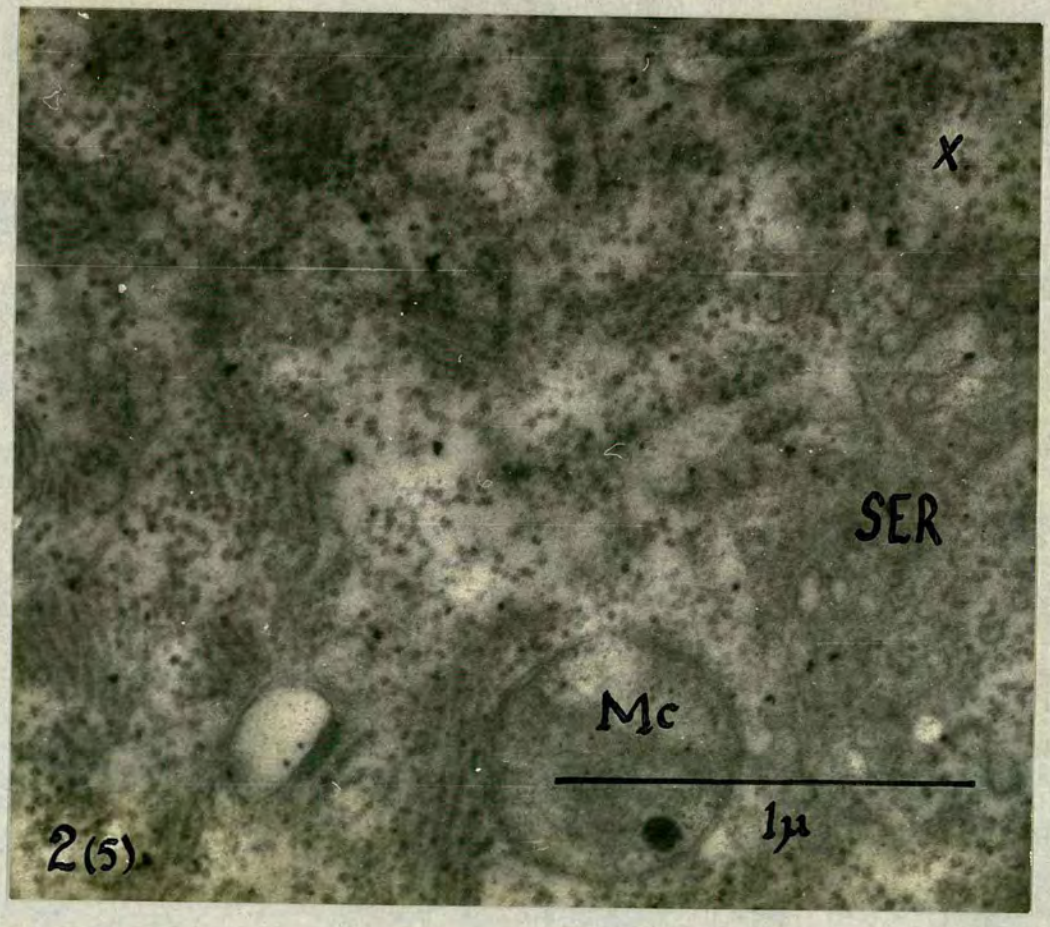
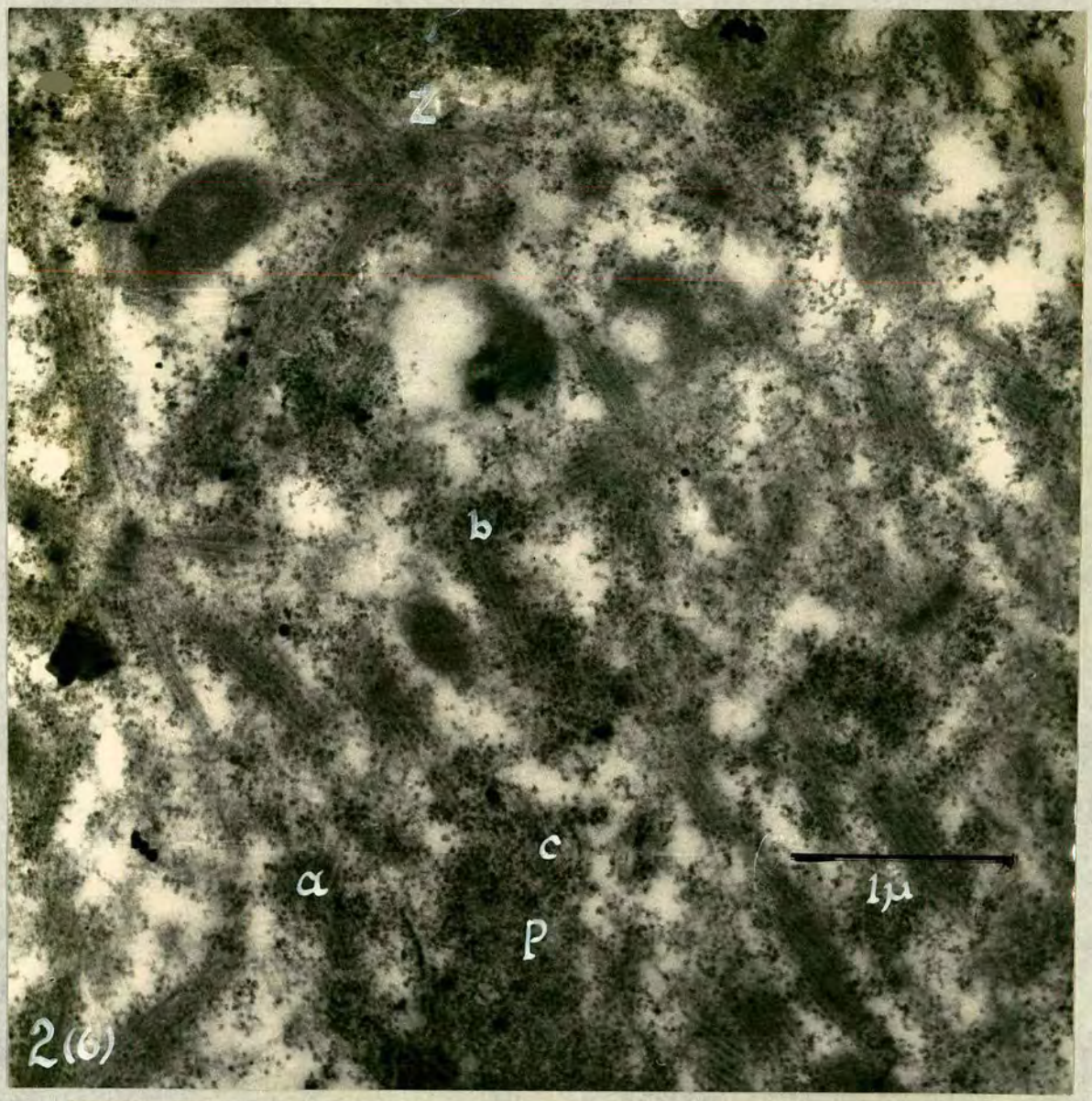
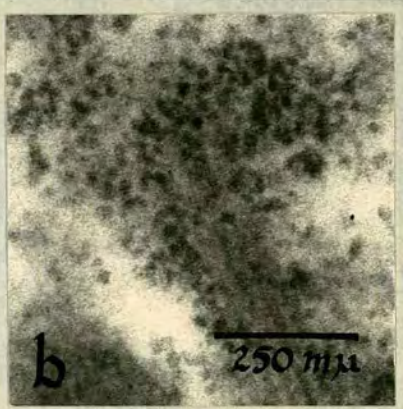


PLATE (5) Minifibrils, including a cross-section (X) Smooth endoplasmic reticulum (SER). Stage 11.



a



b

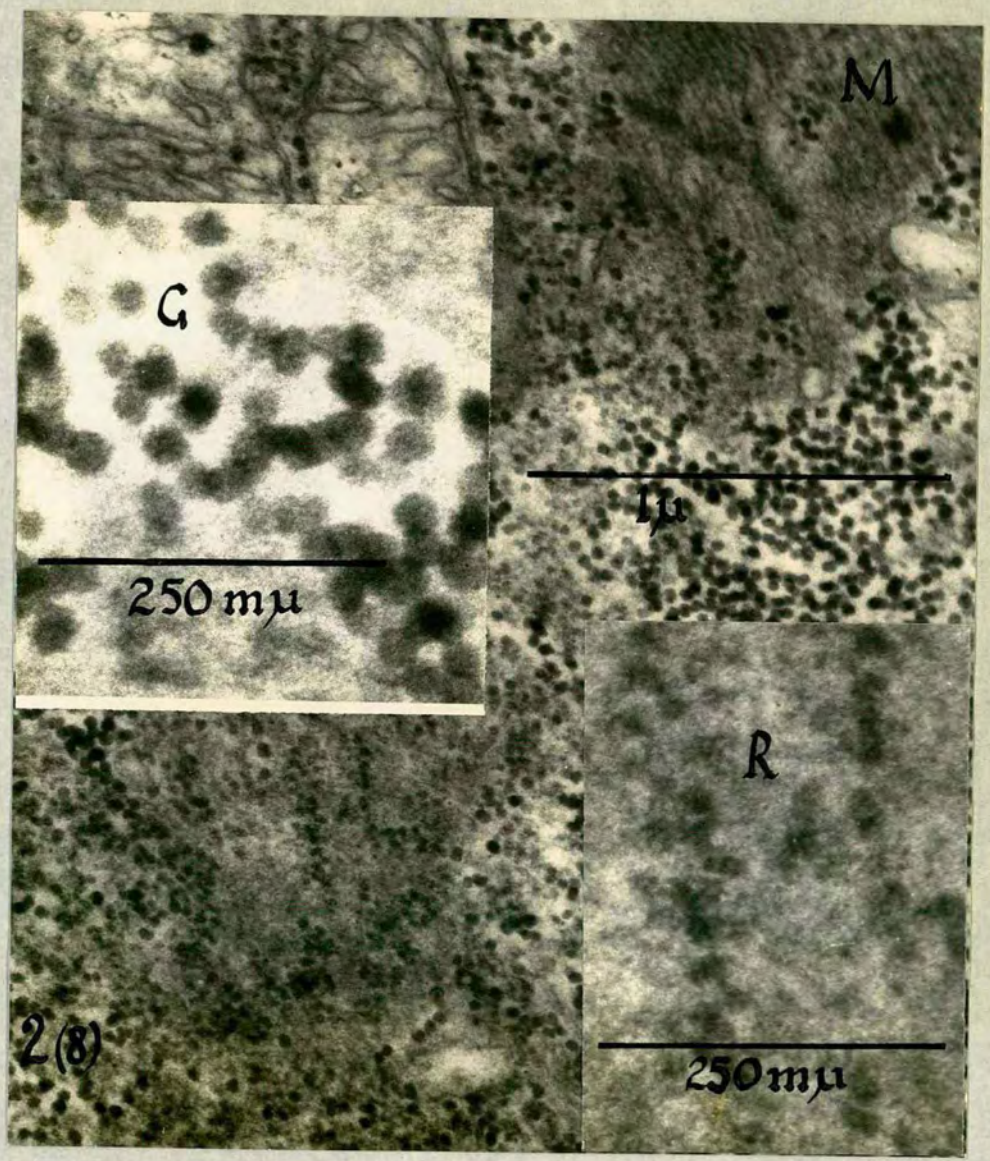
c

PLATE (6). Star-like clusters of minifibrils with Z band material (Z) and helical polysomes associated with myofilaments (b and c). Polysome cluster (P) with helical polysome (c) and closely associated minifibril (a). Stage 13 (neural fold).

PLATE(7). Loose aggregation of orientated mini-fibrils showing thick and thin myofilaments (arrow). Stage 14 (rotation).

PLATE (8). Polysome cluster with ribosomes (inset, bottom right) distinct in size from glycogen granules (inset, top left, same magnification). A mature myofibril is in the same cell (M). Stage 17 (muscular response).

2(7)



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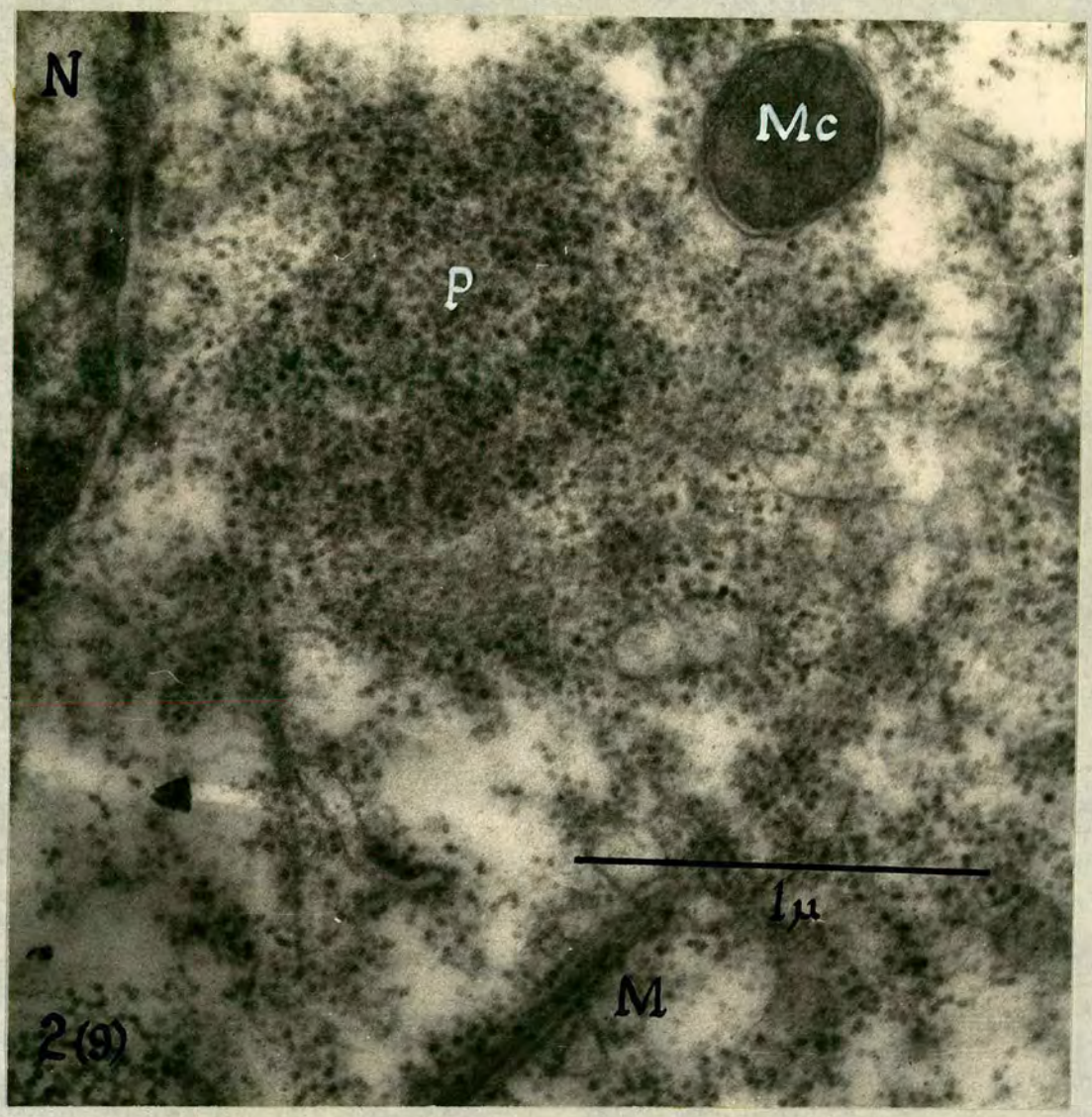
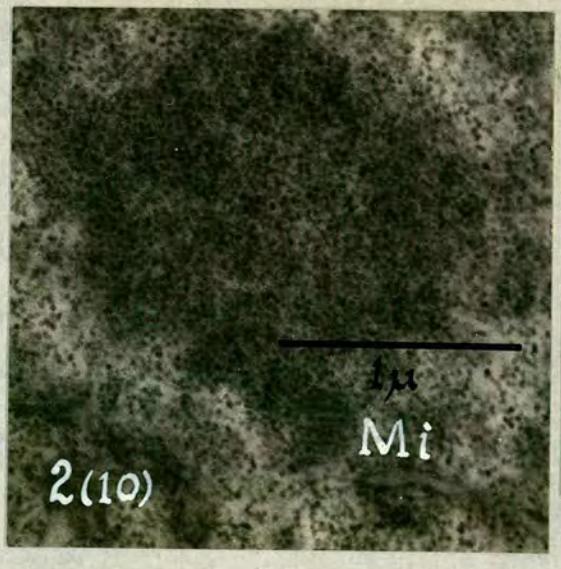
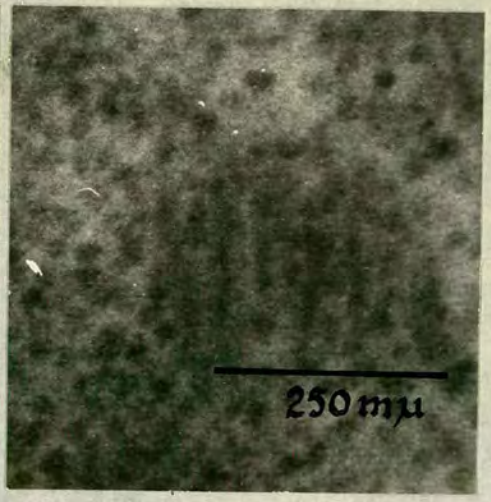


PLATE (9). Polysome cluster (P) and ribosome studded minifibril (Mi). Stage 11 (late gastrula).





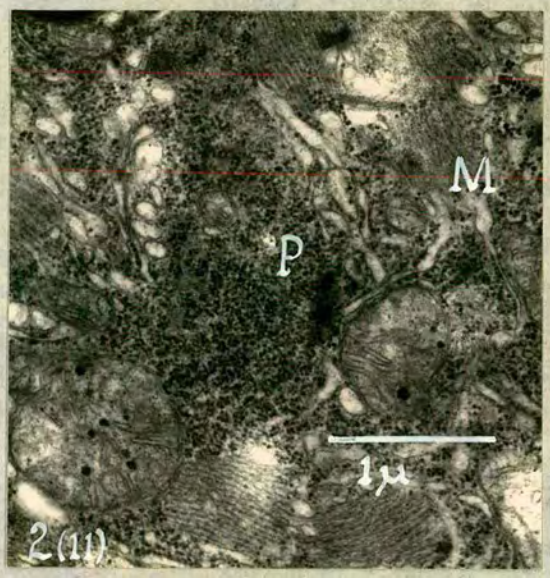
PLATE(10). Polysome cluster with embedded minifibril (enlargement) sectioned obliquely. Stage 11.

PLATE (11). Polysome cluster (P) sandwiched between obliquely sectioned myofibrils (M). Stage 11 (late gastrula).

PLATE (12). Helical polysomes considered to be a myosin synthesizing polysome. Stage 17 (muscular response).

PLATE (13). Amorphouse material (see observation 6). Stage 11 (late gastrula).

PLATE (14). A typical subsarcolemmal region. No skein of thin filaments can be seen. Stage 18 (gill-bud).


M
P
1μ
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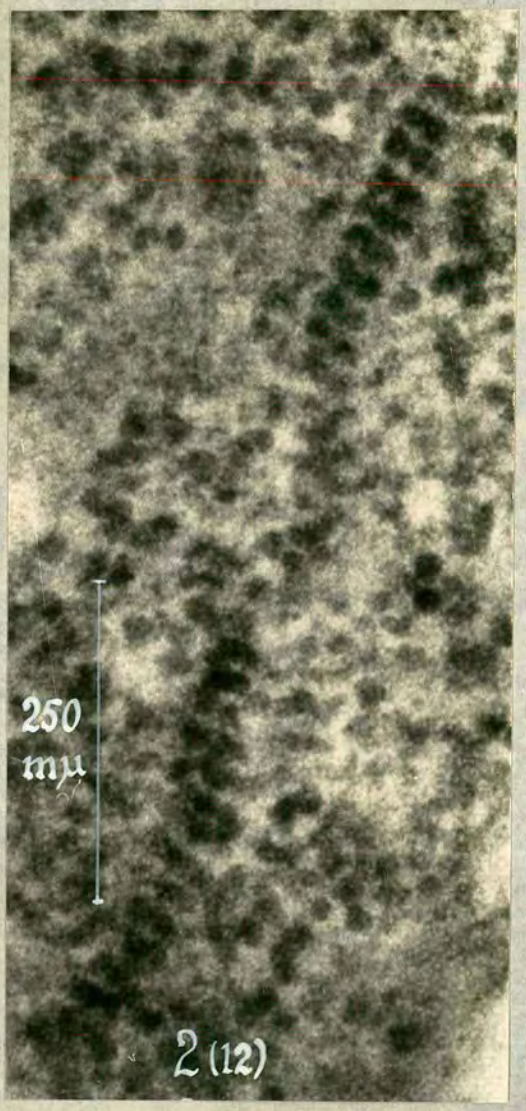
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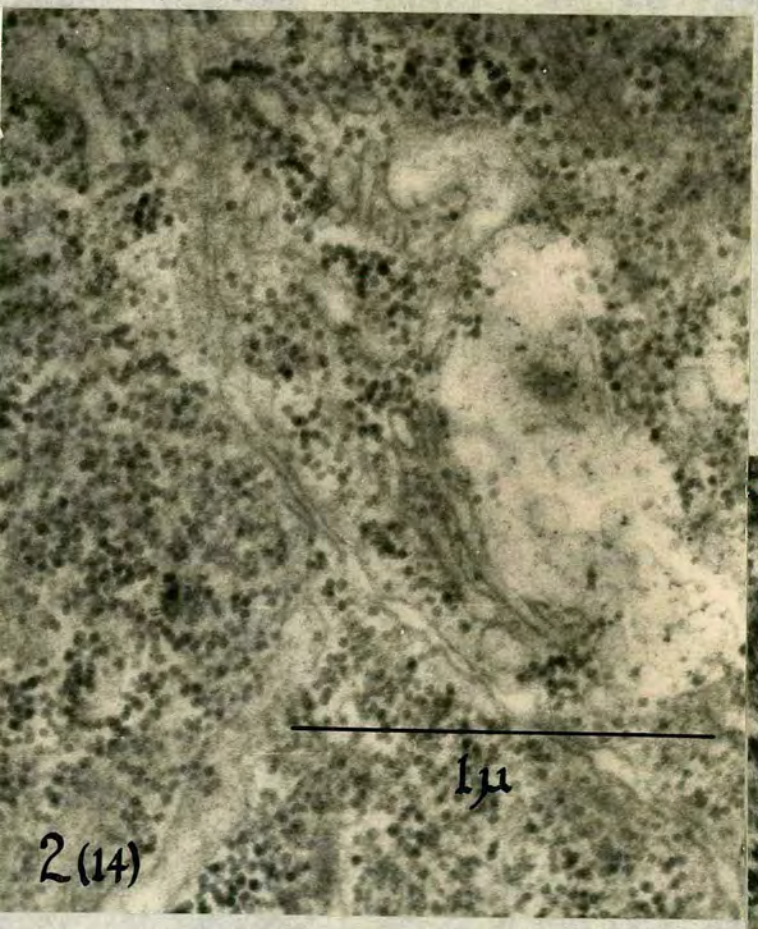
1μ
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1μ
2(13)

B: Observations on Anucleolar (0-nu) embryos:

Essentially the same observations are made on anucleolar embryos as on wild-type embryos.

In all tissues at all stages were seen nuclei with dispersed chromatin, convoluted membranes and blobs, and cytoplasm with large lipid droplets and yolk granules, glycogen, ribosomes, mitochondria and smooth endoplasmic reticulum, (plates 16 and 17). Blobs, replacing the nucleolus, are described and discussed in Chapter 3.1 A(iii)

The special observations are:

(1a) Mature myofibrils (Plate 18) with thick (about 180Å diameter) and thin (about 60 Å diameter) filaments (as measured on Plate 19), and all of the striations and membranous components of the wild-type. Plate 18 shows a long, helical polysome lying alongside the filaments of a myofibril (enlargement). In Plate 27 an oblique section of loosely stacked myofilaments reveals many stretches of helical polysomes. This area has become labelled in a 15 hour pulse with radioactive uridine showing that it contains newly synthesized non-ribosomal RNA.

(1b) Myofibrils with very reduced membrane components, with the striations still in phase (Plate 20).

(2) Minifibrils similar to those seen in wild-type are seen in Plates 15, 21, 22 and 23. In these plates the minifibrils are more densely clustered together than those shown in the wild-type (Plates 4 and 5), but are still randomly orientated except where star-like clusters occur (Plate 22), less obvious than the few seen in wild-type. In the denser clusters of randomly orientated minifibrils, thick and thin filaments are seen stacked alternately in cross sections (Plate 23).

(3) Parallel orientated loose clusters of minifibrils are also seen in O-nu tissue (Plate 24) again containing thick and thin filaments.

(4) Polysome clusters are seen in O-nu tissue in Plates 17, 19, 24, 25. In Plate (17) a long helical polysome is seen lying very close to a polysome cluster. It may well be that the polysomes inside the cluster are of the same sort. Plate (26) shows a clustering of long helical polysome fairly closely packed together but without the amorphous matrix. Perhaps this represents an early stage in polysome aggregation in which synthesis of the protein product has not yet started. The same cluster is shown in Plate (25) and it is seen to be apparently attached to

the end of striated myofibril. This is probably not the case as the myofibril is obliquely sectioned. Plate (19) shows a polysome bundle lying next to the Z band of striated myofibril. Also seen were two similar clusters lying near to the M lines of two adjacent sarcomeres. Plate (24) shows a bundle lying at the side of an orientated loose clusters of myofibrils. Because of the variability of the positions of these polysome clusters it is considered that there is no significance attached to the positions; they are equivalent to freely dispersed clusters, as seen in wild-type tissue. Polysome clusters are seen to take up radioactive uridine in a 15 hour pulse in 0-nu tissue (Plate 24) showing that they contain newly synthesized non-ribosomal RNA. In this plate the cluster is seen to contain polysomes lying in rows roughly parallel to the adjacent myofibrils.

(5) Free polysomes are seen in Plate (15) and appear to be identical to those observed in wild-type tissue.

PLATES (15) - (27). O-NU

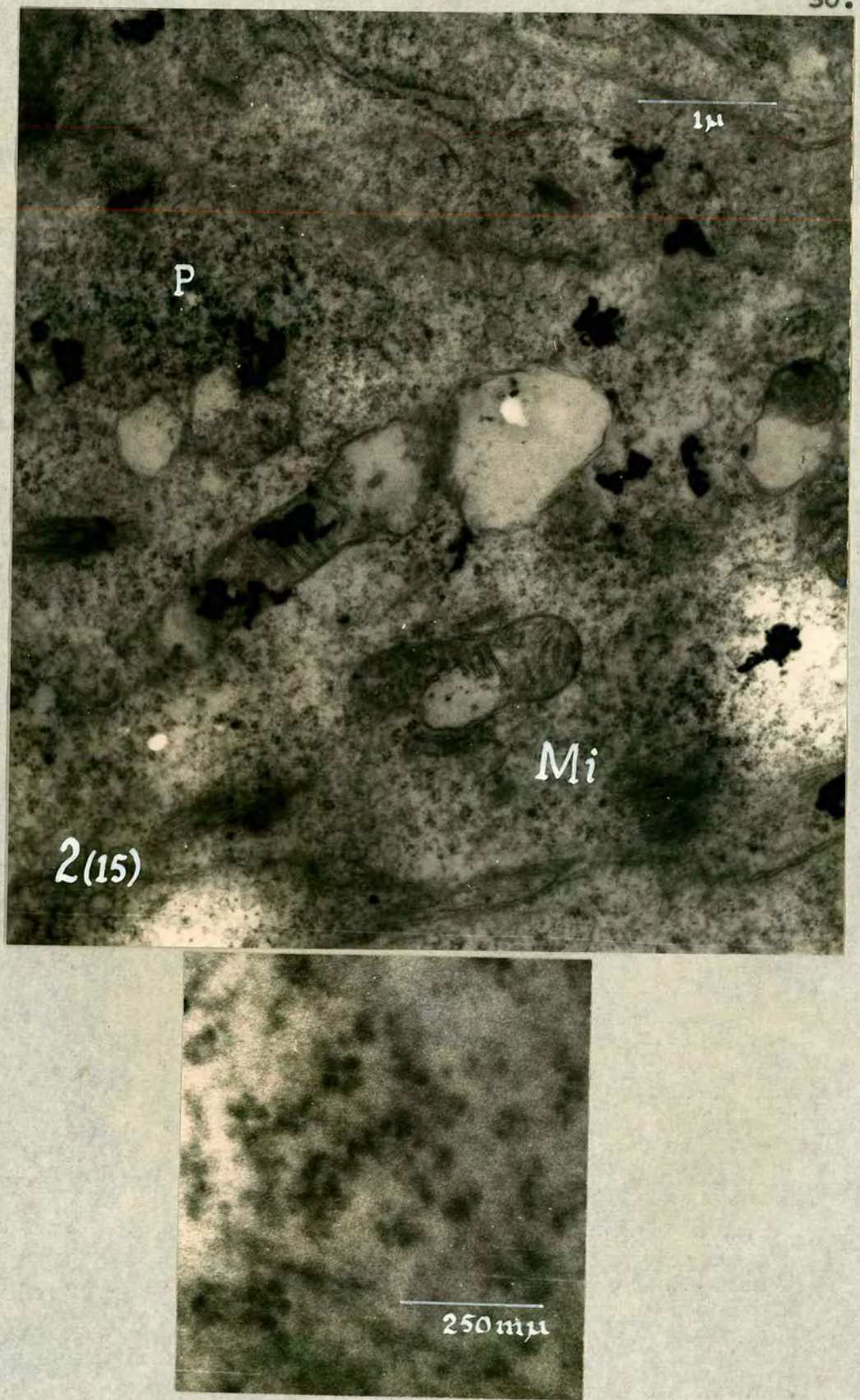


PLATE (15). Minifibrils (Mi) and polysome cluster (P). Many helical polysomes including cross-sections (enlargement). Taken from a 1 hour tritiated uridine pulse autoradiogram (see Chapter 3). The polysome cluster is labelled. Stage 19-20 (hatching).

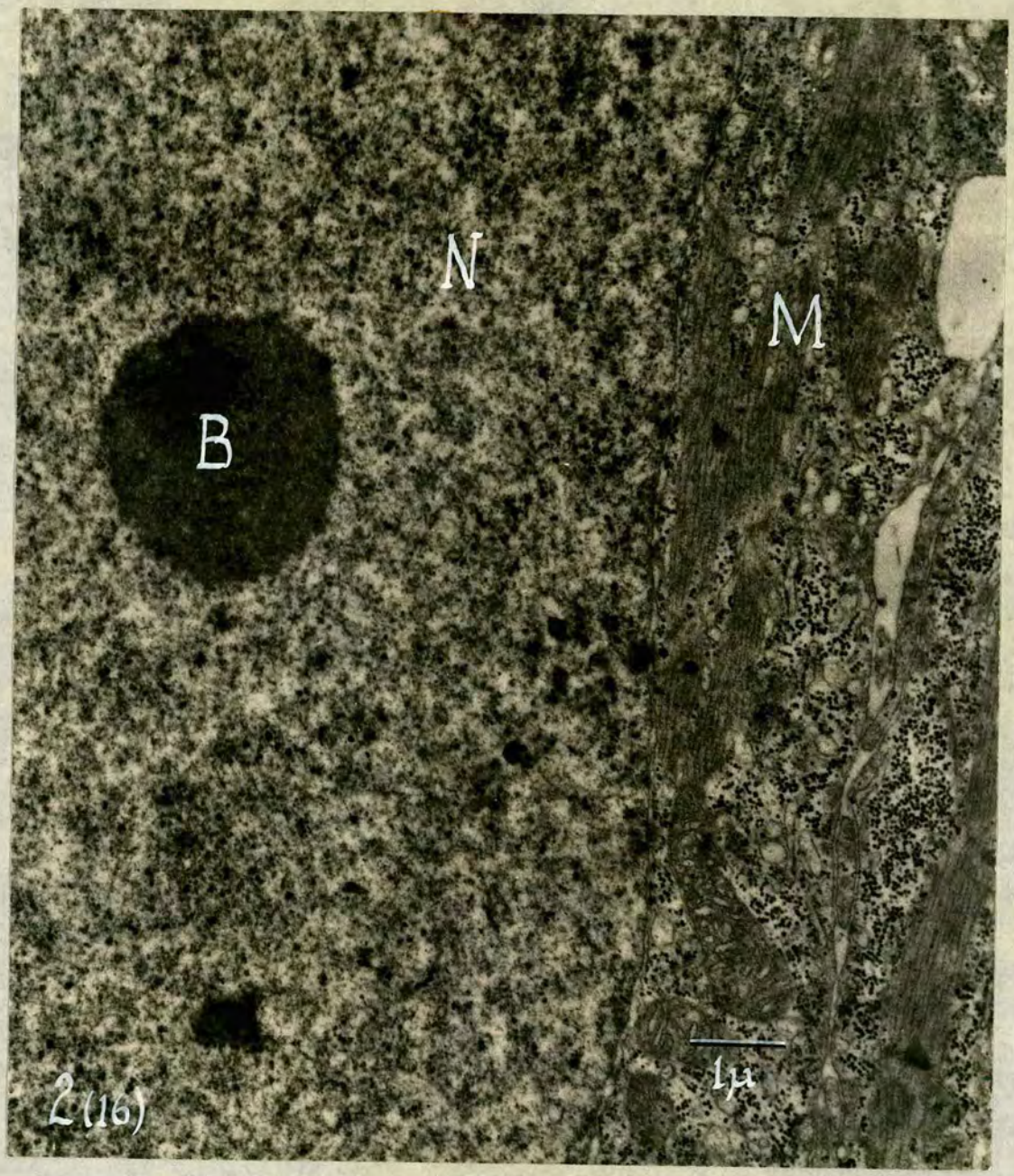


PLATE (16). Nucleus (N), blob (B) and mature myofibrils (M). Stage 17 (muscular response).

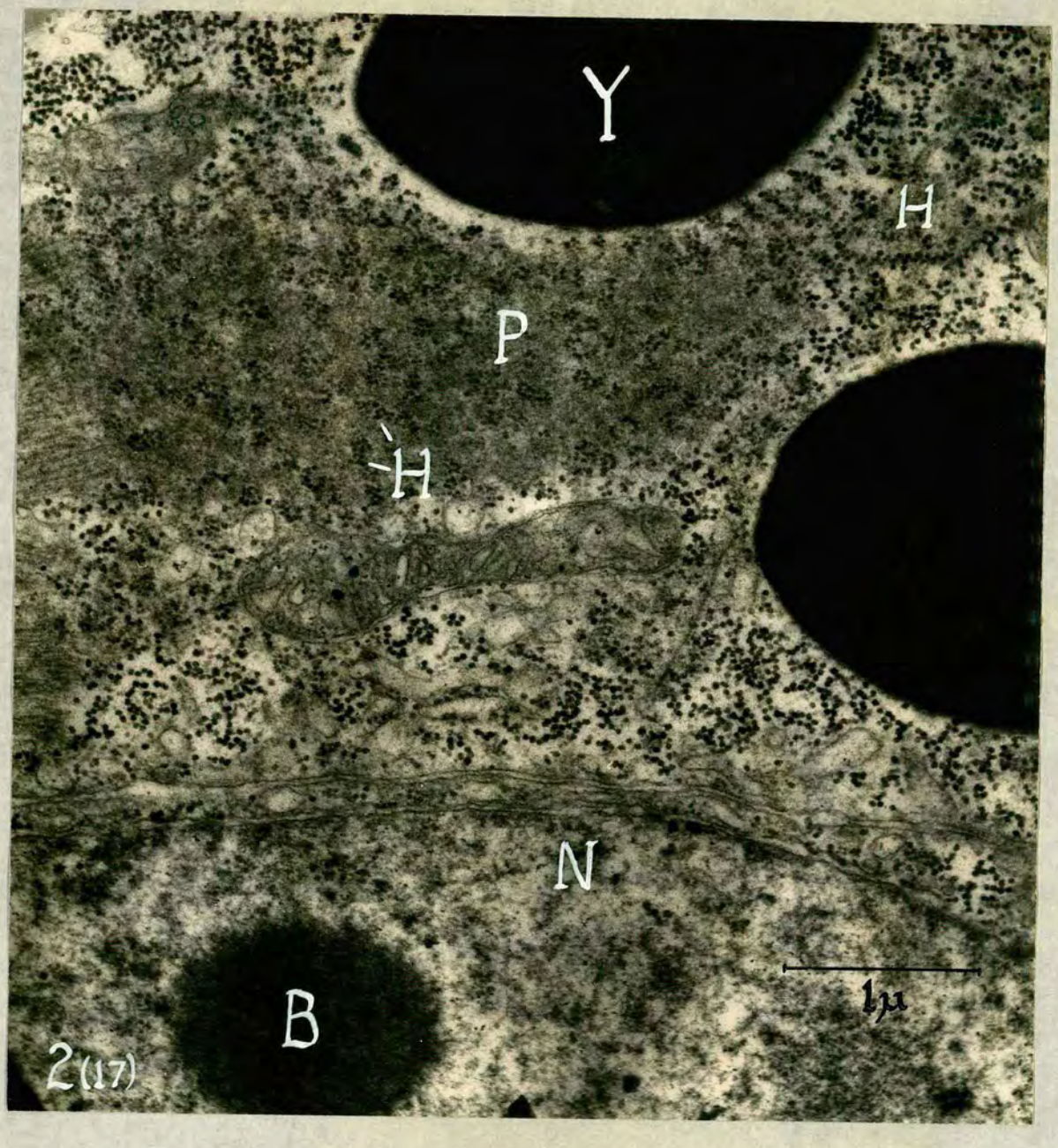
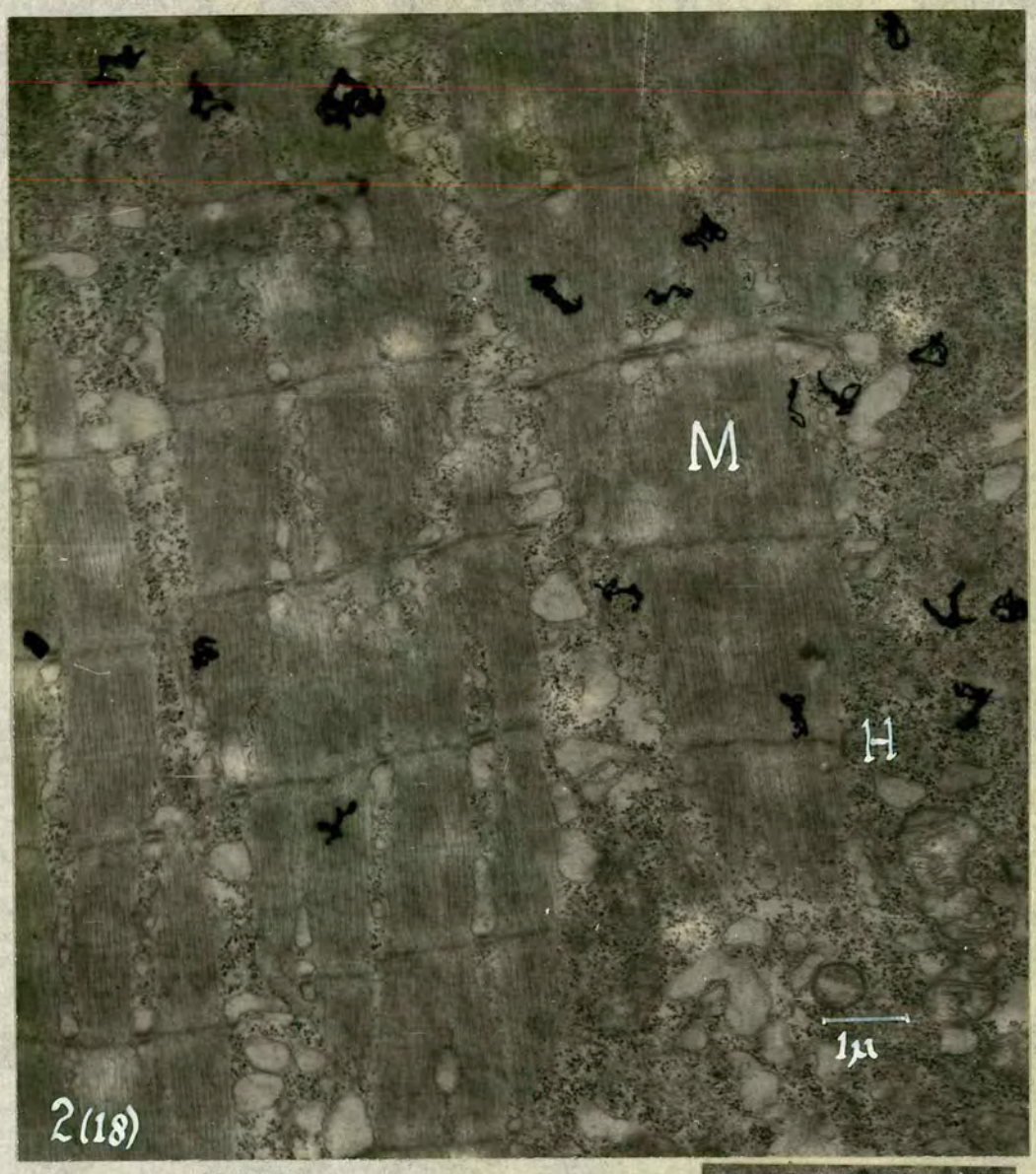


PLATE (17). Polysome cluster (P) with longitudinal and oblique sections of helical polysomes (H). Stage 17 (muscular response).



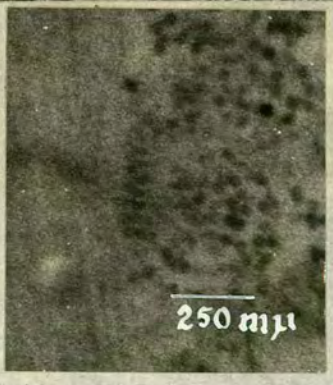


PLATE (18). Mature myofibrils (M). Enlargement shows a helical polysome lying alongside a myofibril. Stage 19-20 (hatching).

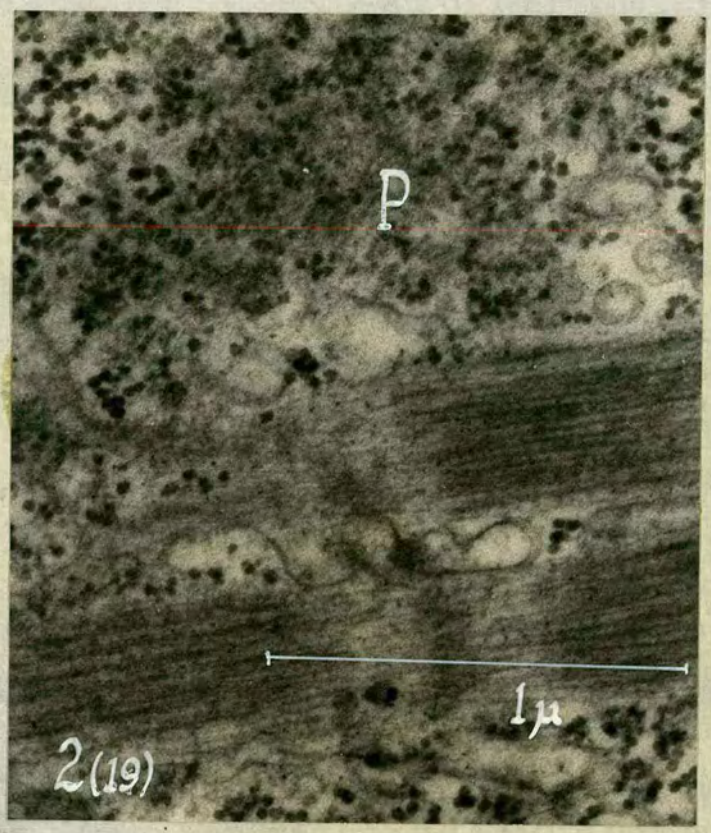


PLATE (19). Polysome cluster lying near a Z band. Stage 17 (muscular response).

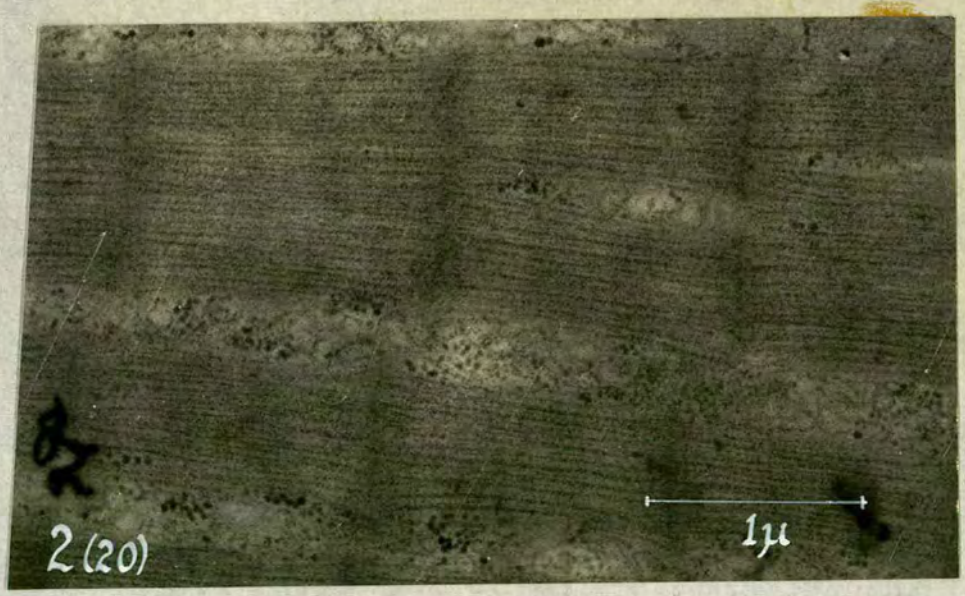


PLATE (20). Striated myofibrils with much reduced sacroplasmic reticulum or transverse tubules. Stage 19-20, (hatching).

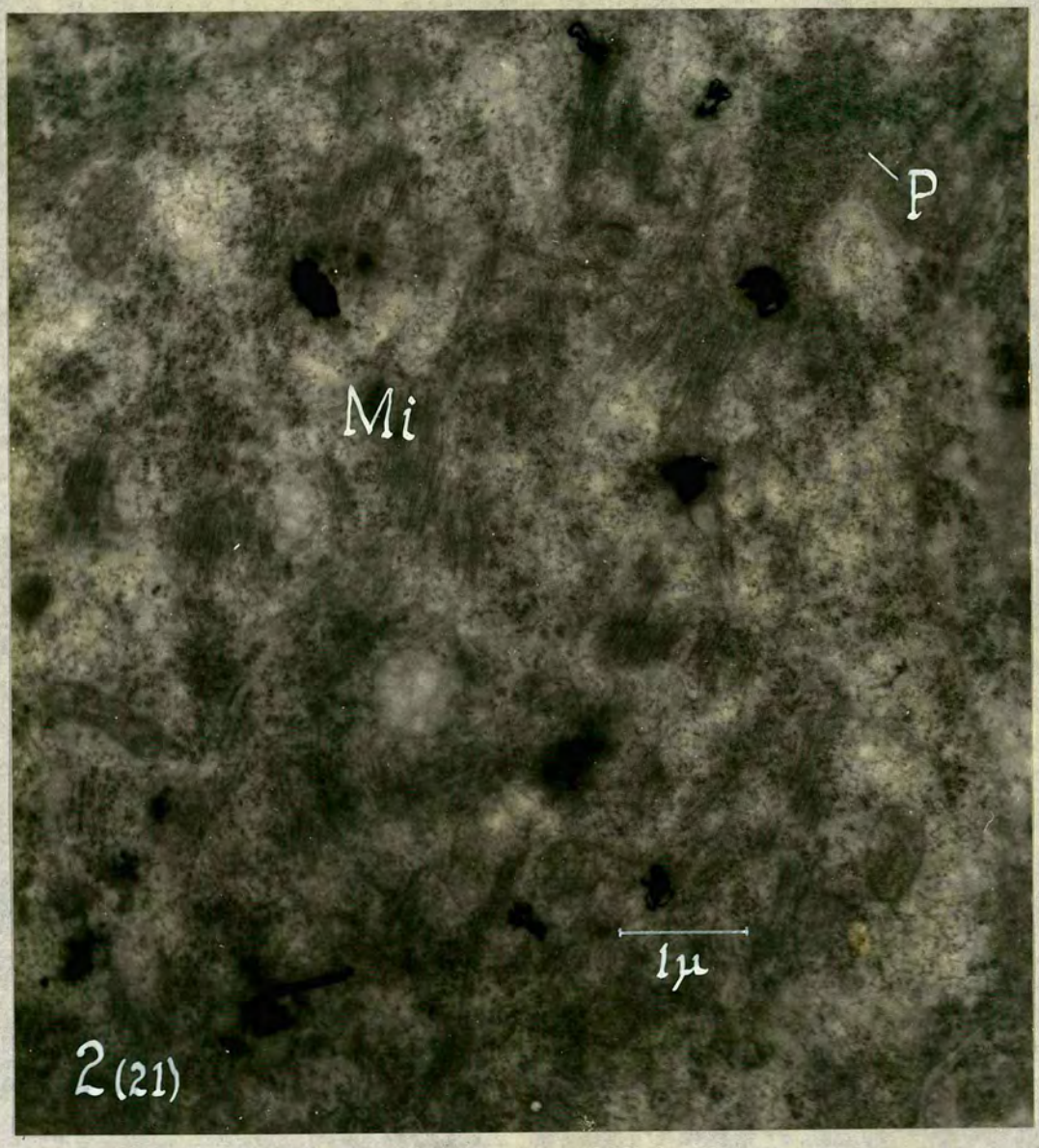


PLATE (21). Minifibrils (Mi), and a polysome cluster (P). Taken from a 15 hour tritiated uridine pulse autoradiogram (see Chapter 3). Minifibrils are labelled. Stage 14 (rotation).

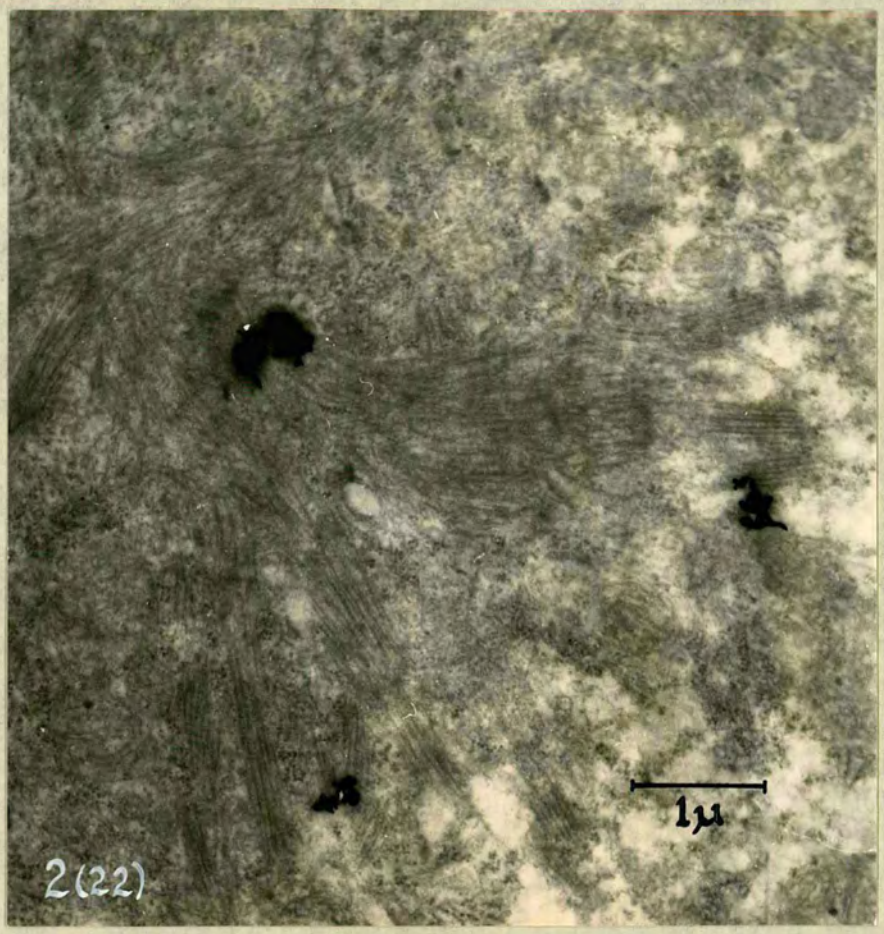


PLATE (22). Disorientated aggregation of minifibrils. Stage 14 (rotation).

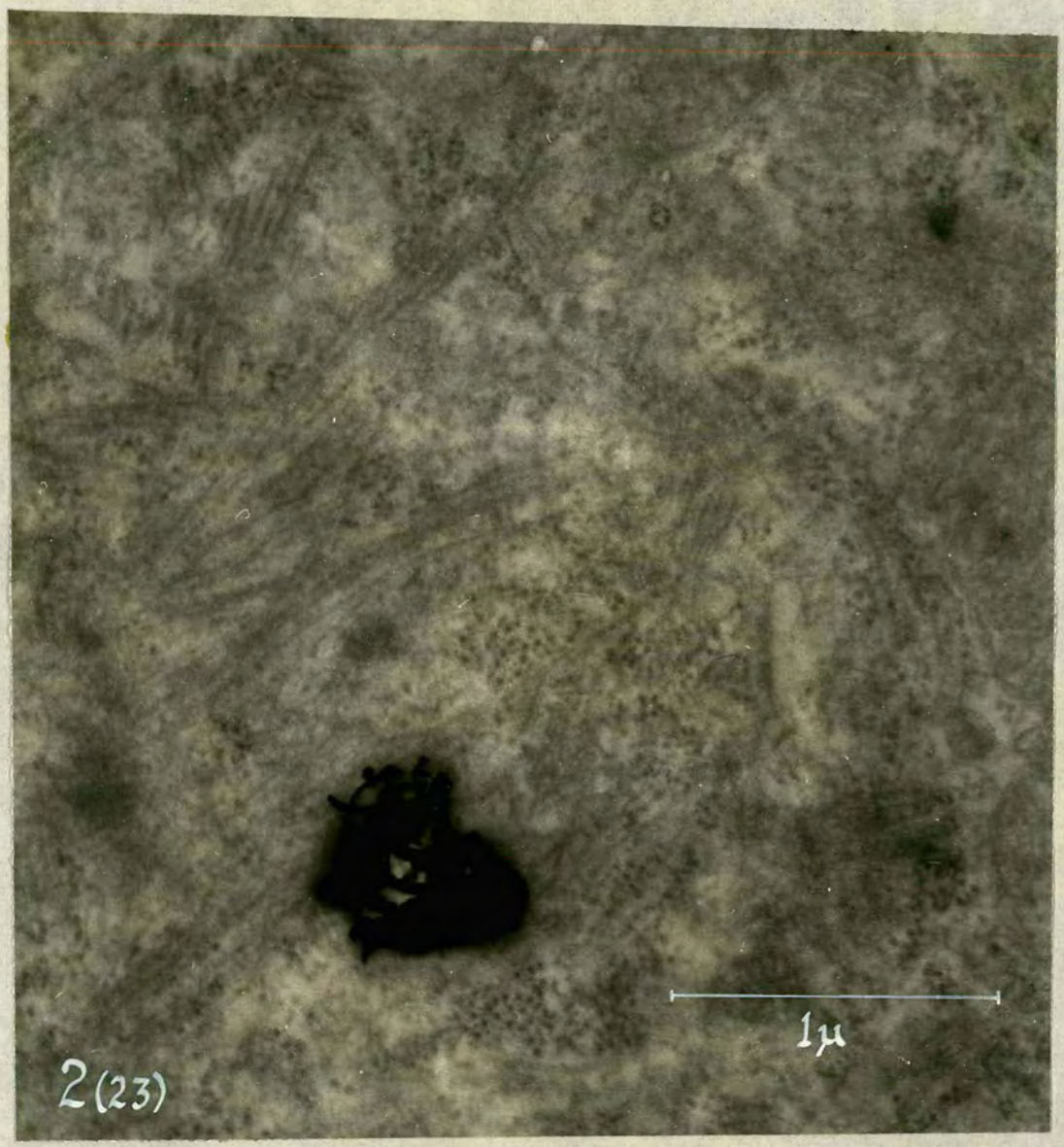


PLATE (23). Disorientated aggregation of minifibrils, including a cross-section. Thick and thin filaments are seen in the cross section. The absence of a transparent centre in the thick filaments shows that they are not microtubules. 15 hour tritiated uridine pulse autoradiogram (see Chapter 3). Stage 14 (rotation).

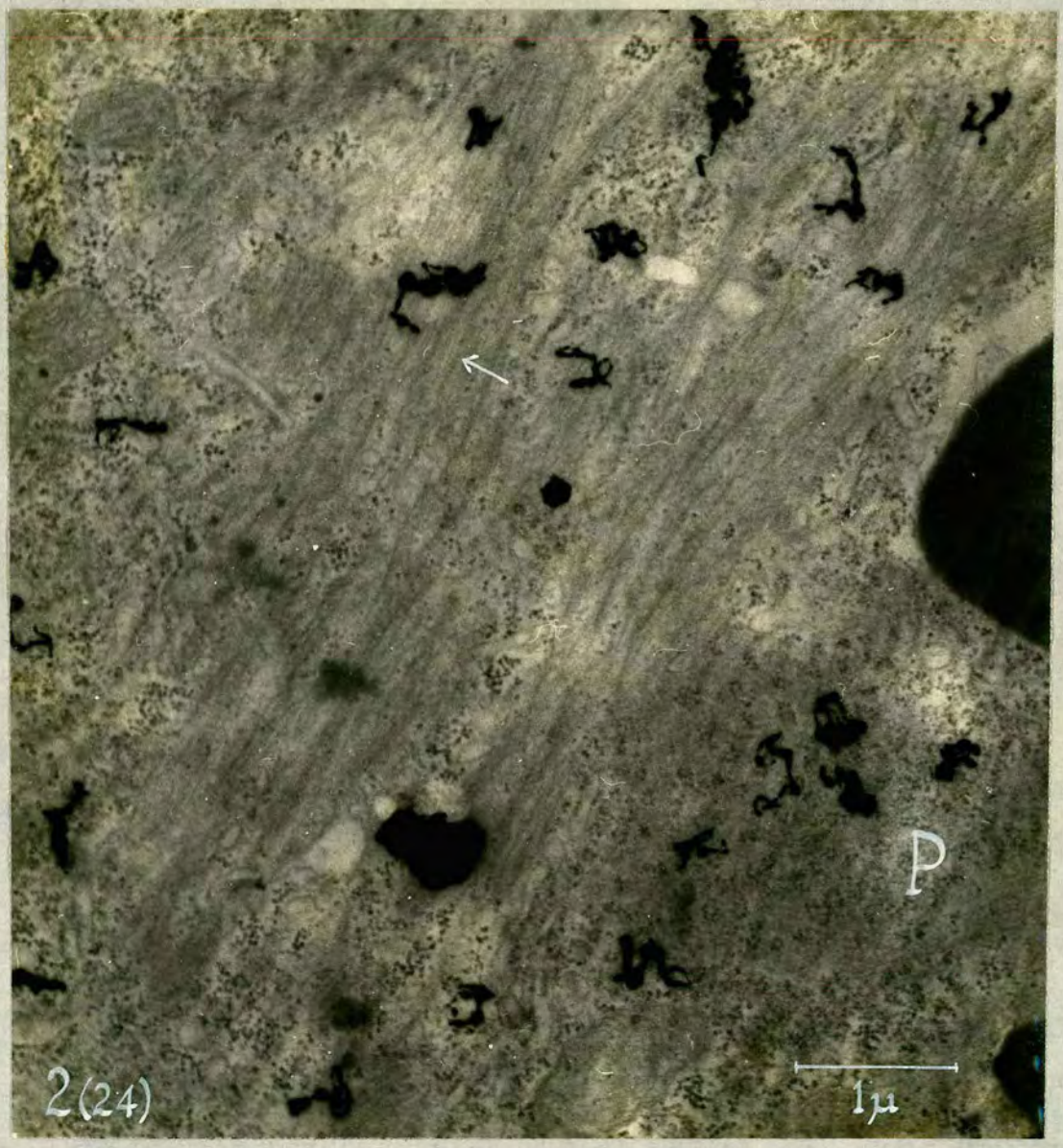
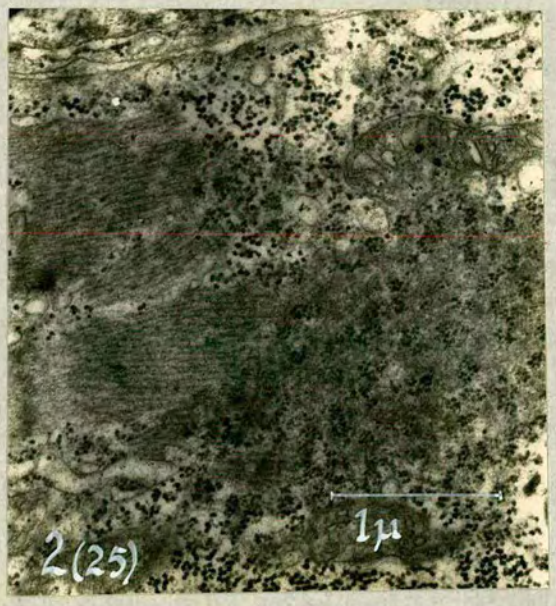


PLATE (24). Orientated aggregation of minifibrils and a polysome cluster (P), from a 15 hour tritiated uridine pulse autoradiogram (see Chapter 3). Arrow shows thick and thin filaments present in minifibrils. Stage 17 (muscular response).

PLATE (25). Polysome cluster situated near an obliquely sectioned myofibril. Stage 17 (muscular response).

PLATE (26). Whorls of helical polysomes. 15 hour tritiated uridine autoradiogram (see Chapter 3). A silver grain is situated close to the whorl. Stage 14 (rotation).

PLATE (27). Helical polysomes (arrows) in an oblique section of loosely stacked myofilaments. 15 hours uridine. Stage 18 (gill-bud).


1μ
2(25)


1μ
2(26)


1μ
2(27)

2.4 DISCUSSION

It is obvious from the results that myogenesis is very similar, if not identical, in wild-type and anucleolar Xenopus embryos. Every structure or combination of structures observed in wild-type is also observed in anucleolar embryos, up to the last stage studied, i.e. stage 20 (early hatching), which is many hours before anucleolar death. Deletion of ribosomal cistrons does not, then affect the morphology of myogenesis in any way observed here. This justifies the use of the animal as a model of normal development in the autoradiographic studies in Chapter 3.

This study has also provided some information about the mechanism of myogenesis. The rest of the discussion refers to both wild-type and anucleolar embryos.

A: Filamentogenesis

Thick filaments are not seen free in the cytoplasm, agreeing with other observations described in the introduction. They are only seen in mature myofibrils and minifibrils. The minifibrils may be free in the cytoplasm or embedded in polysome clusters. The polysome clusters consist of an amorphous matrix containing polysomes which appear to be helical myosin polysomes, often sectioned obliquely giving an

oval shape. Polysome clusters were first described, also in Xenopus embryos, by Moar et al (1971). The polysomes are often seen in rows in the cluster, lying parallel to adjacent minifibrils (e.g. Plate 24). Ribosomes with associated amorphous substance, perhaps nascent protein, are also seen distributed throughout the cytoplasm (Plate 13), but because the ribosomes are irregularly associated in small numbers they are probably not myosin-synthesizing polysomes. This observation probably represents the synthesis of non-myofibrillar proteins. The observation of thick filaments in minifibrils embedded in polysome clusters suggests that the polysomes in the clusters are active in myosin synthesis, and that the myosin polymerizes to form thick filaments whilst closely associated with, if not attached to polysomes. The evidence for myosin also being synthesized in mature myofibrils rests partly on the observations of helical polysomes in, or attached to, myofibrils. Ribosomes are frequently observed in myofibrils by some authors but not by others (see introduction) and in some cases the ribosomes have the helical polysome configuration. This latter observation is repeated here (e.g. Plate 18),admittedly rarely, but on more occasions than those illustrated here. Autoradiographic evidence (Chapter 3) strongly supports the

theory that myofibrillar proteins are synthesized *in situ*.

The evidence then is for there being two different sites of synthesis for myosin; in or on mature myofibrils and in polysome clusters. In both of these sites intact thick filaments are observed. This implies that myosin synthesis and polymerization are closely linked in space, and perhaps also in time. Fischman (1970) argues that because self-assembly of myosin filaments of more or less the correct dimensions has been demonstrated *in vitro* (Huxley 1963 and Kaminer & Bell 1966) by adjusting the pH and ionic strength of a solution of free myosin molecules then there is no need to postulate the involvement of polysomes in this process. He also quotes a similar *in vitro* polymerization of G (globular) to F (fibrous) actin, giving the correct double helix but filaments of lengths different to those found in myofibrils. Polysomes do, however, appear to attach to both types of filament (see introduction), implying that polymerization of nascent monomeric proteins occur. What could be the function of this association if it really occurs? Perhaps the association is passive in that no restriction operates to prevent polymerization before completion of synthesis. Or the association could have a length regulating function, particularly

in the case of actin filaments. Allen & Pepe (1965) reported 'correct' lengths for free actin filaments released from chick embryo myoblasts on homogenization. The association might also play a part in the complexing of actin and myosin with other myofibrillar proteins, the distribution of which is poorly understood, or in fibrillogenesis, discussed below.

Evidence which may be taken as being against the involvement of polysomes in thick filamentogenesis is that the two myosin subunits (heavy and light) are synthesized on separate size classes of polysomes (Sarkar & Cooke 1970 and Low et al 1971) and are apparently synthesized at different rates in vivo (Brivio & Florini 1971). This evidence, however, does not preclude the possibility of the association of more than one type of polysome on a filament. Also Obinata (1969) extracted at an early stage in chick myogenesis an apparently free 3S subunit of myosin in that it was separable from the embryonic 6S (presumably whole) myosin with ammonium sulphate fractionation. The 6S molecule was identified as myosin by its polymerization into complete thick filaments in vitro and its interaction with F-actin. However, at all except very early stages only 6S myosin was found, implying that there is no pool of free myosin subunits during most of myogenesis. The

evidence presented here is for myosin synthesizing polysomes playing a part in filamentogenesis as well as fibrillogenesis, in this case the formation of minifibrils.

As with thick filaments, thin filaments are not seen free in the cytoplasm. They are seen in mature myofibrils and in minifibrils, particularly when the latter are viewed in cross-section. It is not clear if they are present in the minifibrils embedded in polysome clusters although the regular spacing of the myosin filaments suggests that they may be. If not, the observation of non-helically arranged ribosomes associated with minifibrils shows that they could well be synthesized and polymerized in association with myosin filaments. Association of ribosomes with thin filaments has been reported by other workers (see introduction). It is possible that free actin filaments without associated ribosomes do occur but are not observed, for instance because they are few in number or are extremely contorted and dispersed such that only a few scattered portions of thin filaments are present in a section. If this is so then synthesis and polymerization need not be coupled. However, this seems unlikely as the free actin filaments in other organisms are observed as fairly straight filaments (see introduction). It is possible then

that in Xenopus actin is synthesized and polymerized in association with myosin filaments. The probable sequence of events in filamentogenesis is then the synthesis of myosin in polysome clusters in which the polysomes may be orientated in parallel. The myosin monomers polymerize into filaments and stack into minifibrils whilst still in the clusters. Minifibrils are then released from the polysome cluster and occur free in the cytoplasm. Actin is probably synthesized either in the polysome clusters or in minifibrils and is very probably polymerized in situ along-side myosin filaments. Fibrillogenesis then is linked with filamentogenesis in that polymerization and stacking into minifibrils are spacially linked.

B: Fibrillogenesis

Individual free minifibrils probably come together in loose, orientated, non-striated arrays to form the structures shown for example in Plate 24 (observation 3). By this time actin filaments are definitely

present. The next observed stage is the mature myofibril, occasionally with very reduced reticula, implying that reticula possibly form after striation. The only clue to the mechanism of this transition from minifibril to mature myofibril is the appearance of what appears to be Z band material in the centres of star-like clusters of minifibrils. Perhaps the Z band material in some way binds minifibrils together. Z band material is implicated in a similar process by Kelly (1969, see introduction). Unfortunately no other possibly intermediate stages in the formation of striated myofibrils were observed, and so all that can be said is that these stages must be passed through relatively rapidly.

As already discussed myofibrillar protein synthesis and polymerization probably persist in the mature myofibril once it is initiated by aggregation of the minifibrils. Myofibrils grow in width (in the plates shown and in cross-sections, some cells are seen with small myofibrils with large spaces between them whereas other cells are seen with closely packed, wide myofibrils), and as minifibrils are not seen in the close vicinity of mature fibrils it seems unlikely that this growth is due to continued accretion of minifibrils; this seems to be the method of fibril initiation only. The presence of myosin polysomes

in mature fibrils tends to indicate that fibrils grow in width by the lateral addition of individual myofilaments synthesized on polysomes *in situ*. This is less certain for actin than myosin, because actin polysomes cannot be unequivocally identified due to their small size. As previously mentioned free actin filaments are not seen in the cytoplasm. They probably would be seen, if present, immediately before incorporation into myofibrils as it is known where to look for them, i.e. round the edges of myofibrils, and at least some orientation would be expected close to the myofibril. It seems likely then that actin filaments are polymerized and concurrently stacked, if not even actually synthesized *in situ*. The components of the mature fibril could well have a non-passive role as templates for these processes. This then is probably a different mechanism to that taking place in organisms where free thick and thin filaments are seen, as described in the introduction.

To summarise the tentative role of polysomes in the processes leading to the formation of mature myofibrils, it can be said that myosin and perhaps actin polysomes are closely associated with their protein

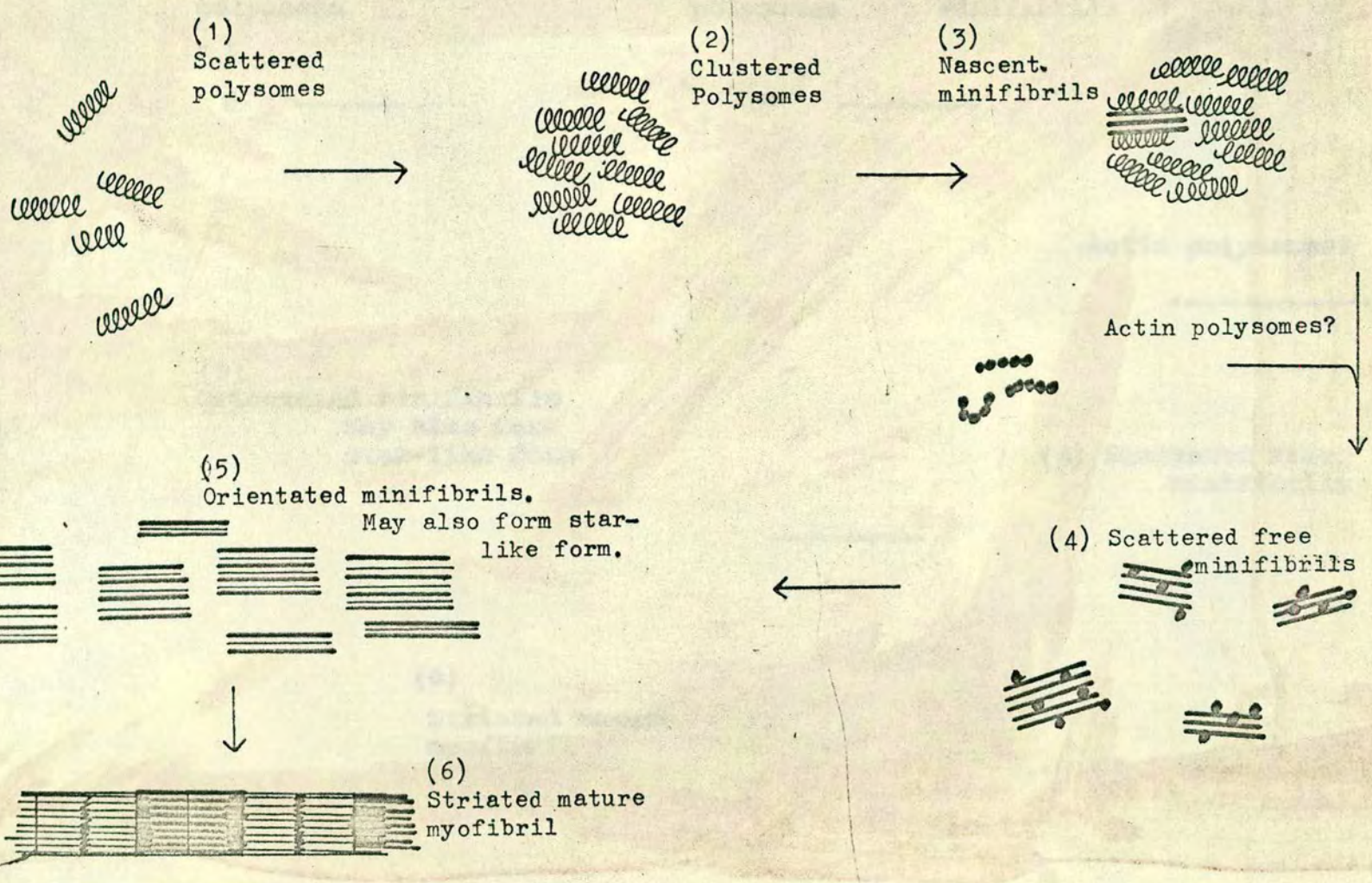
products when these proteins are both polymerizing into filaments and stacking into myofibrils, both in de novo fibrillogenesis and in fibril growth and/or turnover. I suggest that these polysomes may be actively involved in these post-translational stages. This involvement may be exclusive or shared with previously synthesized filaments. I speculate that this involvement is as a template for sterically aiding polymerization and stacking and possibly in the regulation of filament length.

2.5. SUMMARY

(1) Observations on wild-type and anucleolar Xenopus embryos show that myogenesis is morphologically identical in the two cases, so that deletion of ribosomal cistrons does not affect this process in the stages studied before anucleolar death.

(2) The mechanism of myogenesis is reconstructed from these observations. Polysomes are considered to be involved in the post-translational stages of filamentogenesis and fibrillogenesis. A schematic summary is presented overleaf. The findings are discussed with reference to other work in this area.

FIBRILLOGENESIS



CHAPTER THREE

RNA METABOLISM IN WILD-TYPE AND ANUCLEOLAR EMBRYONIC XENOPUS MUSCLE TISSUE:- ELECTRON MICROSCOPE AUTORADIOGRAPHY

3.1. INTRODUCTION

A: Nucleoli

The aspect of nucleolar function most studied is ribosome synthesis, particularly the synthesis of ribosomal RNA (see below i). However, even from its morphology and chemical composition (see Birnstiel 1967 below), the nucleolus contains a greater variety of components than ribosomal RNA and DNA, and ribosomal proteins, and hence presumably has other functions as is discussed below. Much of the work mentioned in this introduction has been extensively reviewed and some of it is referred to a review rather than to the original work.

One aspect of nucleolar function to be discussed in detail is whether or not nucleoli are involved in the processing of messenger RNA or its precursors, because of its relevance in interpreting the results.

(i) The synthesis of ribosomes.

The anucleolar mutant (0-nu) in Xenopus (see Chapter 1) does not possess nucleoli, but instead blobs. The ultrastructure of blobs is described in section (iii) This animal has been shown not to synthesize rRNA (Brown & Gurdon 1964). Neither does rRNA (extracted from cytoplasmic ribosomes) hybridize with 0-nu DNA (Birnstiel et al 1965). They also showed that wild-

type (2-nu) DNA hybridizes twice as well to rRNA at saturation than does the heterozygous (1-nu) DNA. As 2-nu chromosomes have two nucleolar organizer secondary constrictions and 1-nu has only one (Kahn,1962) it follows that the amount of rDNA in the genome is proportional to the number of nucleolar organizers. This has also been shown in a series of nucleolar organizer deletions in Drosophila (Ritossa & Spiegelman 1965). So the part of the chromosome necessary for nucleolar formation [illegible], within the limits of the hybridization technique, is shown to contain all of the DNA coding for rRNA.

When nucleoli are cleanly extracted from pulse labelled HeLa cells and the RNA extracted from them, the label does not, in animal cells, appear as the 18S and 28S RNA of ribosomes on a sucrose density gradient, but in other positions according to the pulse and chase times, but predominantly peaking at 45S and 32S, plus a heterodisperse fraction (Penman et al 1966). The largest molecule present in large amounts, the 45S, does not appear in the nucleoplasm. It was shown that these peaks eventually chase into cytoplasmic 18S and 28S RNA in the ribosomes. Also Perry (1962) showed that a particular concentration of the RNA synthesis inhibitor actinomycin D (AMD) specifically inhibited the appearance of label in cytoplasmic rRNA

and nucleolar RNA in L cells. The nucleolar 45S and 32S are, then, precursors of rRNA.

This is good evidence for the nucleolus being the site of transcription and partial processing of rRNA precursor from the DNA template. A more rigorous review of the evidence for this is presented by Birnstiel (1967), who also presents the evidence for some further steps in ribosome biogenesis, namely methylation of the RNA and its complexing with ribosomal proteins also taking place in the nucleolus. Liau et al (1970) have detected and separated methylation activities acting on nascent preribosomal RNA and exogenous tRNA in isolated rat nucleoli. Warner & Soeiro (1967) have extracted RNP particles from HeLa nucleoli and shown that they contain some of the rRNA precursor molecules, showing that proteins associate with preribosomal RNA in the nucleolus. Birnstiel (1967) states that isolated nucleoli can incorporate amino acids into proteins, some of which have an amino acid composition similar to ribosomal proteins, but it has not definitely been shown that ribosomal proteins are made in the nucleolus. It is not known where another ribosome component, 5S RNA is synthesized. 5S RNA, (Monier et al, 1969) is found in some nucleoli, (see Birnstiel, 1967) and has been shown to be present in the nucleolus at least shortly after synthesis (Warner & Soeiro 1967 and

Sirlin et al, 1966). Nucleoli and the nucleoplasm probably contain different isozymes of the enzyme DNA dependent RNA polymerase. Probably the nucleolus contains the isozyme which preferentially transcribes rDNA as opposed to bulk DNA. Isolated nuclei have been used in studies where the different forms of the enzyme are activated and inhibited by manipulating salt concentrations and the products characterized as ribosome-like or DNA-like RNA. Simultaneously the cellular location of the different activities were checked by autoradiography. The r-DNA preferring polymerase I was active in the nucleolus and the bulk-DNA preferring polymerase II was active in the nucleoplasm and not the nucleolus (Widnell & Tata 1964; Maul & Hamilton 1967; Pogo et al 1967). RNA polymerase has been extracted from rat liver nuclei (Roeder & Rutter 1970) and analysed. Nucleoplasmic polymerase is predominantly polymerase II and nucleolar polymerase is predominantly polymerase I. These extracted polymerases respond to salt concentration manipulations as do the autoradiographically defined polymerases of the previous authors. These preferences for different templates are determined by differences in some of the subunits of the polymeric enzyme. A factor conferring specificity was extracted from E.coli polymerase and characterized by Burgess et al (1969) and Travers (1969). This was found to be one of the enzyme sub-units which could be dissociated

from the enzyme (Burgess et al 1969; Travers 1969; Avila et al 1970). This subunit is called the sigma factor. A factor ψr which preferentially stimulates rRNA synthesis has been extracted from E.coli (Travers et al 1970) which will probably turn out to be a sub-unit of RNA polymerase I. In higher organisms, sigma factors have been extracted from amphibian oocytes (Crippa et al 1970) and Xenopus embryos (Roeder et al 1970), which also shows the presence of polymerase I in 0-nu embryos. The evidence from polymerase studies suggests then that the principal nucleolar function is rRNA synthesis. The synthesis of nucleolar RNA has been studied autoradiographically at both the light and electron-microscopic level. Some of the more recent work, which attempts to distinguish between fibrous and granular region labelling is now considered. Amphibian oocytes and pre-gastrula embryos have pre-nucleoli, also called primary nucleoli. These are usually entirely fibrous in nature and are smaller and more numerous than mature nucleoli and are similar in appearance to the blobs found in anucleolar Xenopus nuclei. Prenucleoli develop granular regions and thus become mature nucleoli at around the gastrula stage, when rRNA synthesis commences (Brown & Littna 1964). Karasaki (1965) found in an electron-microscope autoradiographic (EMARG) study in Triturus (newt) embryos that a 3 hour pulse of tritiated uridine (3HU)

labelled the chromatin surrounding the prenucleoli. Mature nucleoli labelled in the boundary between the fibrous and the granular regions. After a 21 hour chase in unlabelled medium the granular region also labels. By this time the cytoplasm is also labelled. From this he concluded that nucleolar RNA is synthesized in the chromatin associated with the fibrous region and later passes via the granular region to the cytoplasm. Essentially similar results were obtained with the sea-urchin Arbacia (Karasaki 1966). Granboulan & Granboulan (1965), using EMARG, found that a 5 minute pulse of 3HU labelled only the fibrous region of the nucleolus in monkey kidney cell cultures. After only a 10 minute pulse the granular region was also labelled. La Cour & Crawley (1965), again using EMARG found that in Ipheion root meristem a 1 hour pulse of 3HU labelled only the central nucleonema of the nucleolus although it is not clear from their photographs if this region is entirely fibrous. Fakan & Bernhard (1971), using EMARG with monkey kidney cells, found that a 2 minute pulse of 3HU labels perinuclear chromatin and that a 5 minute pulse is required to label the fibrous regions of the nucleolus. A 15 minute pulse is required to label the whole nucleolus. A 3 hour chase does not eliminate nucleolar labelling. This general concept of the synthesis of nucleolar RNA in the fibrous region or in the chromatin

in its periphery, and its transport to the granular region before exit from the nucleolus is supported in other work. Sirlin et al (1961), using light microscope autoradiography (LMARG), on the nucleolus attached to a giant chromosome of a Chironomid larva found that the nucleolus organizer (inserted into the fibrous region of the nucleolus) labels first with radioactive ribonucleosides, followed by the nucleolar periphery (where the granular region is). These findings were confirmed by EMARG by Gaudecker (1967). In contrast to the findings of Karasaki (1965), MacGregor (1967) found that Triturus oocyte nucleoli do label uniformly with 3HU as seen by LMARG. In addition, at a later stage where a granular region forms on the side of the prenucleolus facing the centre of the nucleus rather than the nuclear membrane, as observed by EM, LMARG shows that 3HU pulses of up to 2 hours label the side of the nucleolus facing the nuclear membrane, i.e. the fibrous region. In longer pulses the nucleolus is uniformly labelled. Lane (1967) made the same observations.

Most of these workers claim that because the earliest structure in the nucleolus to label is the fibrous region and in others the nucleolus associated chromatin, then the observed label represents ribosomal precursor RNA and that this precursor is synthesized in the

fibrous region or the nucleolus associated chromatin. Subsequent appearance of label in the granular region then represents, and shows the site of, precursor processing. The argument rests mainly on analogy with previously discussed biochemical evidence, even though the organisms and the time scales are not necessarily the same. Das et al (1970), however, almost proved the argument directly by doing biochemistry and autoradiography in the same experiment using Urechus (worm) isolated nucleoli. Unfortunately the autoradiography was done at the light microscope level and so the words 'core' and 'cortex' have to be used instead of 'fibrous' and 'granular' regions. They labelled the core only, with a 2 hour pulse and extracted a large, labelled ribosomal precursor RNA. A pulse of 4 hours labelled the whole nucleolus and the extract showed a partially processed precursor. Chasing for 6 hours with AMD after a 2 hour pulse labelled the cortex only and gave on extraction an even more highly processed precursor. Unfortunately this does not distinguish labelling of the core region only from labelling of the inner part of the granular region. Thus, being pessimistic, one could argue that there is no clear cut case for the fibrous region being involved in rRNA production at all; this could be synthesizing an entirely different kind of RNA, the autoradiographic

appearance of which is masked by labelling of the inner cortex with rRNA precursor.

This is a general criticism of the LMARG work previously discussed. Also the sucrose gradient analyses of Das et al (1970) show, in addition to the ribosomal precursor peaks, a small, heavy peak at the bottom of the tube and some heterodisperse material across the rRNA - 4SRNA region. This material, although less in total activity than the ribosomal precursor peaks could, if concentrated, give higher autoradiographic labelling than the ribosomal species.

Phillips & Phillips (1971) treated mouse and chinese hamster cell cultures with toyocamycin which is known to prevent the processing of 45S rRNA precursor without preventing its synthesis. EM showed that the nucleoli had lost all of their granules and were totally fibrillar. No labelling was done in these experiments. This could be taken to show that the fibrillar region consists of non-processed 45S RNA. However, the anucleolar mutant heterozygote of Xenopus does not synthesize 45S RNA and also contains fibrillar bodies, or blobs in the nucleus. This supports theories developed from the results of this chapter that the pars fibrosa has functions other than rRNA transcription. This aspect is more fully introduced in section (iii) below.

It is clear, then, that rRNA originates in the nucleolus, that the pathway involves the granular region and that most of nucleolar RNA synthesis is of rRNA or its precursors. What is less clear is how much of the labelling appearing in the ARGs, using tritiated uridine, represents rRNA and its precursors. A species of RNA synthesized at a slower rate, or in a smaller mass per nucleus, than rRNA precursor would still be observed in ARGs if it occupied a smaller volume than rRNA precursor such that labelling of this slowly synthesized RNA gave a higher specific activity in Curies per volume of tissue. In fact if the volume were small enough the minor species would show up as silver grains in a shorter time of pulse than would rRNA precursors. In this case the conclusion that the first site to show labelling is the site of rRNA precursor synthesis would not be valid. There is ample evidence for the nucleolus being involved in the metabolism of RNA other than ribosomal precursor, and this is discussed below. The anucleolar Xenopus does not synthesize rRNA (Brown & Gurdon 1964) and yet it possesses nuclear blobs which are identical in EM sections to nucleolar cores (Jones 1965).

The results to be presented show that in wild-type Xenopus nucleoli, the pars fibrosa and the pars

granulosa have independent labelling kinetics with tritiated uridine. No precursor-product relationship is apparent.

(ii) Non- ribosomal nucleolar functions.

As previously mentioned, the ultrastructure of the nucleolus reveals many constituents, some of them presumably being non-ribosomal because not all of them occur in all cells, whereas rRNA is probably synthesized or capable of being synthesized in all cells.

The presence of vacuoles of various sizes, some containing granules and fibrils, large 300 Å granules in addition to the 150-200 Å granules thought to be ribosome precursors, crystaloids, haemoglobin, protein, lipid and carbohydrate inclusions, membranes, chromatin lamellae and dense 'spots' in normal and pathological nucleoli of various plants and animals are reviewed by Birnstiel (1967), Hay (1968) and Bernhard & Granboulan (1968).

Birnstiel (1967) mentions several cases of nucleoli containing 4S transfer RNA (tRNA) including that of Vincent et al (1966) which shows the presence of 4S RNA in starfish oocytes. When chromosomal RNA synthesis is 90% inhibited in Smittia salivary glands with substituted benzimidazoles as checked by LMARG the remaining nucleolar RNA synthesis includes tRNA

in addition to 5 and 28S RNA. This tRNA is also methylated in situ and both methylation and synthesis are inhibited by AMD showing that nascent tRNA is probably methylated in the nucleolus (Sirlin et al 1966). Interestingly this method of blocking chromosomal RNA synthesis by 90% also reduces nucleolar labelling by nearly 30%. In the same organism Sirlin et al (1961) showed by LMARG that pseudouridine is taken up by the nucleolus. This is a base unique to tRNA. In contrast Perry (1962) showed that 4SRNA is synthesized only in the nucleoplasm of L cells, and that this subsequently chases into the cytoplasm. Woods (1966) came to the same conclusion using LMARG in Vicia faba meristems. Here total RNA was labelled with 3H-cytidine. The material was then fixed and some of it was extracted with a salt solution which he showed selectively removed tRNA. ARGs of both samples were prepared and the difference in grain intensity gave the amount of tRNA synthesis. This was zero for nucleoli, but this would be expected if tRNA were only a small percentage of the total nucleolar RNA synthesis and if in addition to synthesis of tRNA, end turnover of CCA groups in tRNA was masking the picture, which he claims may be the case. Wallace (1966) claimed that labelling in anucleolar Xenopus pulsed with 3H-uridine would represent tRNA synthesis as Brown & Gurdon (1964) showed that this

was the major species of RNA synthesized in this animal. However these authors show there to be some hetero-disperse RNA also synthesized, which may contain messenger RNA (Gurdon & Ford 1967). The fact that anucleolar mutants make tRNA does not necessarily mean that the nucleolus is not involved in rRNA synthesis; the blobs and associated chromatin and indeed even some of the non-associated chromatin in anucleolar nuclei could represent scattered components of the normal nucleolus, since Wallace (1962) showed that heterochromatin, all of which is associated with the nucleolus in wild-type, is considerably more dispersed in the mutant. However, perhaps because of the low resolution of LMARG, Wallace did not see any clustering of silver grains in O-nu nuclei after 3H-uridine or methyl-C14-methionine pulses, which may have represented blob labelling. Wild-type nucleoli did, however, have more grains than the nucleoplasm when methyl-C14-methionine was given with puromycin to stop protein labelling with this compound,showing that the nucleolus is involved in RNA methylation, some of which was tRNA methylation. Birnstiel et al (1963) showed that isolated pea nucleoli methylate exogenous tRNA. The methylated product also contains some 8-14S RNA of unknown function but presumably originating from the nucleolus. Liau et al (1970) have extracted from isolated rat nucleoli methylation

activity specific for exogenous tRNA. The balance of evidence then is for some nucleoli synthesizing and methylating tRNA.

Several enzymes, some not necessarily connected with rRNA or tRNA synthesis, have been detected in isolated pure nucleoli, often at higher concentrations than elsewhere. Siebert (1966) demonstrated RNA polymerase, RNAse, NAD pyrophosphorylase and ATPase activities in isolated rat liver nucleoli. Some of these which were also found in the nucleoplasm differed from the nucleoplasmic form in substrate specificity, state of inhibition and response to drugs. It seems unlikely that nucleoli would contain enzymes, particularly enzymes with unique properties, unless they had a function.

Nucleoli are active in protein synthesis. Many cases of amino acid incorporation into proteins by nucleoli, detected for instance by autoradiography, are cited by Hay (1968) and Birnstiel (1967), who states that some newly synthesized proteins are in the residual fraction of nucleolar proteins which also contain some ribosomal proteins, but that the products of nucleolar protein synthesis have not been sufficiently characterized. Vincent et al (1966) showed that

starfish nucleoli contain many non-ribosomal proteins but it is not known where they are synthesized.

There is substantial evidence both for and against some nucleolar RNA being synthesized in the nucleoplasm. It is very important that this is fully investigated because it could provide information on part of the mechanism of cell differentiation. The most promising place to look for a control of differentiation is in the various steps of protein synthesis, because a cell is largely determined by what proteins, including enzymes, it possesses. Theoretically protein synthesis could be controlled at any of its steps; i.e. DNA replication, mRNA transcription, mRNA processing and transport and translation on the ribosomes. Controls have been demonstrated at each of these steps in various organisms. The passage of RNA from the nucleoplasm to the nucleolus prior to its passage to the cytoplasm could represent mRNA processing, provided of course that it could also be demonstrated that some of this RNA contained mRNA or its precursor, that the nucleolus actually processed it in some way, that some of it reached the cytoplasm and that it was in fact translated. Even this would not necessarily involve a control of differentiation unless it could be shown that this process operated selectively on specific mRNAs. The reason why processing

of mRNA has been considered as a control process arises from the dilemma in which people who look for mRNA in nuclei find themselves. Thus Penman et al (1968), finding that most nucleolar RNA synthesized in HeLa cells is ribosomal precursor RNA, looked for RNA having the characteristics of mRNA in the nucleoplasm. The bulk of nucleoplasmic RNA synthesis, observed either by selectively blocking rRNA synthesis with a low dose of AMD (Penman et al 1968) or physically separating the nucleolus from the nucleus (Penman et al 1966) and extracting RNA from pulse labelled cells, is into so called heterodisperse nuclear RNA (HnRNA). This has a heterogeneous sedimentation pattern of 10-90S (many overlapping size classes are present), is like bulk DNA in its base composition (ie. it does not contain much rRNA which has a high GC content compared with bulk DNA), and it has a high turnover rate. Cytoplasmic RNA (non-ribosomal, as observed in short pulses of radioactivity) is again heterodisperse in sedimentation and a small part of it is associated with polysomes. Polysomes, when extracted whole from pulse labelled cells, carry radioactivity at 10-70S which maintains its sedimentation characteristics when the polysomes are disagregated in EDTA showing that the actual RNA which is labelling, or a complex of this RNA with

protein has a sedimentation coefficient of 10-70S, overlapping in size with the naked extracted HnRNA. In fact the labelled RNA extracted from HeLa polysomes is 10-30S (Penman et al 1970), overlapping with but smaller than HnRNA. When a radioactive pulse is chased with a high dose of AMD nuclear and cytoplasmic RNA both decline in specific activity, the nucleoplasmic RNA most rapidly. This led Penman to the disturbing conclusion that HnRNA is not a precursor of cytoplasmic mRNA. What may be more likely is that the nuclear pool of mRNA or precursor is so small (HnRNA containing a lot of RNA not destined to become mRNA) that the cytoplasmic mRNA specific activity rises during a chase but rapidly falls before the first measurements are made. Other work (Soeiro & Darnell 1970) shows that HeLa HnRNA and polysomal mRNA competitively hybridize with HeLa DNA which shows that they do have common sequences. However, in experiments in which RNA is recovered from the hybrids and rehybridized (Darnell et al 1970) it was shown that the apparent overlap in sequences is in reiterated parts of the molecules and this type of rapid hybridization may not be highly sequence specific. This work showed that HnRNA had more reiterated sequences than mRNA. Scherrer et al (1970) in a search for the 'missing link' which would show if HnRNA was a precursor of

mRNA assayed various cell fractions for ability to incorporate amino acid into proteins, having shown that all fractions had all of the necessary subsidiary factors by demonstrating their ability to incorporate amino acid into proteins with added poly-uridylic acid (synthetic message). In the absence of poly-U they found protein synthesizing activity, and hence presumably mRNA, in polysomes and a sub-ribosome fraction of HeLa cytoplasm, but not in the monosome fraction. This sub-ribosome fraction then contains RNA with at least one property in common with mRNA: it can direct the incorporation of amino acids into proteins. When fixed in formalin after selectivity labelling non-ribosomal RNA using a low dose of AMD this fraction yields a radioactive peak on a caesium chloride density gradient at 1.45g/cm^3. This is the same density as the RNP released from polysomes by EDTA and the same as the peak containing all of the radioactivity in the pulse labelled post-chromatin fraction of nuclei. This 1.45g/cm^3 particle is by nature of its extraction and buoyant density an RNA-protein (RNP) complex. When this messenger-like RNA is extracted from the post-ribosomal fraction it competitively hybridizes to DNA with polysomal mRNA and with HnRNA at very high Cot values indicating that they probably contain some common non-reiterated sequences. When the kinetics of HnRNA were studied Scherrer et al

(1970), in agreement with Penman (1968) found that there was no overall reduction in size with chasing. Instead, all size classes simultaneously decay although the larger molecules decay faster. Strangely no RNA smaller than 10S was found in the chase experiments showing that some kind of orderly degradation was taking place, probably by breaking large molecules into still large pieces, but it not known where the pieces go. If HnRNA is a mRNA precursor it seems likely that some of the smaller pieces travel to the cytoplasm (polysomal mRNA is of a smaller range of sizes than HnRNA), probably immediately after scission, as the smaller size classes do not accumulate in a chase experiment. A similar state of affairs exists in rat liver cells. Henshaw (1968) showed similar 30S rapid labelling particles extractable free in the cytoplasm, and released from polysomes by EDTA. The particles have a buoyant density of 1.45g/cm^3 and the rapid label RNA is unlike rRNA in base composition. An apparently identical particle was isolated from rat liver nuclei by Samarina et al (1968). This particle contained most of the rapidly ($\frac{1}{2}$-1 hour) labelled RNA which had a DNA like base composition and had a buoyant density of about 1.40g/cm^3. When extracted with an RNAse inhibitor present polymers of this particle up to 12 units long (as observed in EM) were isolated. These could be converted back to the

30S monomer with RNAse. These authors envisaged chains of these globular proteins forming on mRNA thus protecting it from degradation and assisting its transfer to the cytoplasm. The 30S particles do in fact bind to added DNA-like RNA, not rRNA. Spirin & Nemer (1965) discovered 1.45g/cm^3 RNP particles in the sub-ribosome fractions of the cytoplasm of fish and sea urchins. These particles stimulated free ribosomes into protein synthesizing activity. They label rapidly but do not themselves, unlike heavy polysomes, synthesize proteins. They were, then, thought to be particles which contain mRNA and which, on binding with ribosomes, form active polysomes and which were called informosomes. In none of these cases demonstrating cytoplasmic RNP particles with informational properties has it definitely been demonstrated by labelling kinetics that they originate from the nucleus. If this were the case it would be difficult to demonstrate kinetically, e.g. by a decline in nucleoplasmic specific activity during a chase experiment coupled with a rise in informosome specific activity if only a small part of the nucleoplasmic RNA was an informosome precursor and if there was only a very small

pool of the precursor in the nucleus, as would be the case if the precursor immediately migrated to the cytoplasm on formation, such that there would be only a very brief rise in specific activity in the cytoplasm during a chase. However, as the evidence stands, nuclear origin of messenger RNA is not yet proven.

If HnRNA is a precursor of mRNA several processing functions must take place, mRNA is on the whole smaller in average size than HnRNA. The HnRNA molecules must either be reduced in size (the scission being presumably at rather precise places) or the smaller molecules must be selectively transported across the nuclear membrane. This scission or selective transport must also involve a reduction in the number of reiterated sequences. The mRNA, at some stage in its production, must also bind to protein. This may overlap with the other processing activities. This association must be rather specific in order to render the particle able to attach to ribosomes so as to form active polysomes. As not all of the HnRNA goes to form mRNA (the evidence above is for a small part, if any of it doing so) then the portion destined to do so must be somehow labelled as different from the rest. This labelling may be wholly intrinsic in that the base sequence entirely determines that it alone eventually forms

polysomes, or else the sequence may trigger the aquisition of a different kind of label, e.g. the type of associated protein, or some entirely different type of processing. Even if HnRNA is not a precursor of mRNA it is difficult to imagine that it is not important to the cell in some other way, and so its processing, even if for an unknown function, is still interesting. This processing may not involve the nucleolus but this is a possibility.

What then is the evidence for nucleoli at least taking up some of the RNA made in the nucleoplasm? One piece of evidence is surprisingly direct. Rho & Bonner (1961) labelled isolated pea nuclei with tritiated cytidine and extracted RNA separately from the nucleoli and nucleoplasm. Their results show that nucleoli only label after the nucleoplasm has labelled and that in a chase experiment the nucleoplasmic specific activity declines whilst the nucleolar activity rises, indicating that at least some nucleoplasmic RNA travels to the nucleolus. Goldstein & Eastwood (1966) retracted an earlier claim from autoradiographic experiments with human amnion cells that all of nucleolar RNA is derived from the nucleoplasm after being unable to repeat this result. They did not say if they had disproved that even part of newly synthesized nucleoplasmic RNA chases to the nucleolus.

Amano & Leblond (1960), using quantitative LM-ARG and colorimetric estimation of RNA content of 3H-cytidine labelled DNAsed and stained sections of mouse tissues obtained specific activities of RNA (in grains /mass of RNA) in chromatin, nucleolus and cytoplasm. Their results show a crossover point in specific activities of nucleolus and cytoplasm during a chase. The curve for whole nuclei does not cross the other curves. This they claim shows that all cytoplasmic RNA derives from the nucleolus. This is undoubtedly true for ribosomal RNA which, being the predominant species, will have the most influence on their results, but the results do not eliminate the possibility of a minor species of RNA travelling from the nucleoplasm to the nucleolus. In fact the results are not inconsistent with the theory that all nucleoplasmic RNA destined for the cytoplasm travels through the nucleolus. Perry et al (1961), using a similar technique with HeLa cells showed that UV irradiation of individual nucleoli in intact cells apparently completely abolishes nucleolar labelling and reduces cytoplasmic labelling to 2/3 of the level found when an equivalent area of nucleoplasm is irradiated, which causes a 1/3 drop in labelling of the cytoplasm compared with the non-irradiated control. Again this is consistent with some nucleoplasmic RNA passing

through the nucleolus on the way to the cytoplasm but Perry's kinetic evidence shows that nucleoplasmic labelling is not sufficient to account for all of the label appearing in the cytoplasm. This would not have to be the case if the nucleolus had a dual role of synthesizing rRNA and processing nucleoplasmic mRNA. However they conclude that most or all of the nuclear RNA reaching the cytoplasm comes from the nucleolus and that all nucleolar RNA is synthesized in situ. Their judgement seems to be biassed by the predominant ribosomal RNA species observed in the autoradiograms. The data does show that irradiation of the nucleoplasm does cause a fall in nucleoplasmic labelling. Other evidence on these lines includes Karasaki's (1966) finding (already discussed) that nucleolus associated chromatin was the site of initial labelling, the label spreading to the rest of the nucleolus later. Penman et al (1966) shows that although ribosomal precursor RNA forms a large part of the newly synthesized RNA in HeLa cell nucleoli there is a tail of heterodisperse RNA. Thus although the autoradiographic evidence could represent tRNA travelling to the nucleolus this work shows that some other type of RNA is metabolized in the nucleolus. It is not shown in Penman's data whether this RNA is synthesized in or chases into the nucleolus. As previously mentioned Sirlin et al (1966) showed autoradiographically that inhibiting

chromosomal RNA synthesis by 90% also reduces nucleolar labellin by nearly 30%. This could however be interpreted as a direct action of the drug on nucleolar RNA synthesis.

The main clue that this non-ribosomal nucleolar RNA may be of great significance in genetic expression comes from the work of Henry Harris' group at Oxford on hybrid animal cells (Harris 1968). Harris has for many years campaigned the belief that mRNA and ribosomal 'structural' RNA are the same thing. Whilst not wholly supporting this view I think that some of his experimental findings have some significance here. The techniques consist of fusing together cells in culture, of different species and tissues of origin, with inactivated Sendai virus to form heterokaryons which effectively contain the nuclei of both types in the cytoplasm of one, and then observing the formation in the cytoplasm of substances specific to the cell type of the 'foreign' nucleus. Chick erythrocytes do not contain anything resembling a nucleolus. When the nuclei of these cells are introduced into human or mouse cells (Harris et al 1969), hen specific antigens appear on the surface of the human or the mouse cell and within a couple of days they disappear. Presumably this is a carry over of unstable antigens from the cytoplasm of the chick cells. Later on hen specific antigens appear on the surface of the

heterokaryon and accumulate to high levels, after the formation of nuceoli in the chick nuclei. Similarly, when a chick erythrocyte nucleus is introduced into a mutant mouse cell lacking an emzyme, the enzyme is synthesized in the heterokaryon cytoplasm, as shown autoradiographically by the incorporation of radioactive substrate, again at the same time that the chick nucleus produces a nucleolus, (Harris & Cooke 1969). It is unfortunately not clear if actual synthesis of the antigen is taking place or some kind of activation of preformed substances. The conclusion reached by Harris is that the antigen and enzyme can only be synthesized when the messenger-ribosome is synthesized. Autoradiography and centrifugational characterization show that the chick nuclei are active in heterodisperse RNA synthesis at all times. Ribosomal precursor peaks appear when the nucleolus becomes visible which coincides with the possible synthesis of hen specific substances in the cytoplasm. It may be that a nucleolar function is necessary to transport mRNA from the nucleus. In order to test this hypothesis some rather inconclusive experiments were performed (Sidebottom & Harris 1969) in which individual nuclei in the heterokaryons were irradiated with UV light. Mouse cell whole nuclei were first irradiated with UV and the number of silver grains in the nucleus and cytoplasm, after a pulse of 3H-uridine and autoradiography,

was drastically reduced compared with non-irradiated controls. This shows that irradiated mouse nuclei contribute little or no RNA to the cytoplasm. When a mouse nucleus was irradiated in a heterokaryon also containing 3 or 4 chick nuclei 1 to 3 days after fusion when the chick nuclei did not possess nucleoli there was virtually no labelling of the cytoplasm although the chick nuclei were labelled. Thus anucleolar chick nuclei synthesized RNA but transported little, if any of it into the cytoplasm. When mouse nuclei are irradiated in 7 day old heterokaryons in which chick nuclei have large nucleoli, pulsing with 3H-uridine does label both the heterokaryon cytoplasm and the chick nuclei. Thus chick nuclei bearing large nucleoli do manage to transport a lot of RNA to the cytoplasm. At this time hen specific substances are detected in the heterokaryon cytoplasm. Although these findings support the theory that nucleoli process mRNA such that it is transported into the cytoplasm they are not conclusive. Small amounts of mRNA could continuously be passing into the cytoplasm from chick nuclei and coming under some kind of translational control. The increase in cytoplasmic labelling attributable to chick nuclei at the nucleolar stage could represent purely ribosomal RNA. Also it could be that the simultaneous appearance of nucleoli in the nucleus and hen specific substances in the cytoplasm

is fortuitous: perhaps the genes are not transcribed until a time when nucleoli also happen to form. A better experiment would be to irradiate all of the chick nucleoli in a heterokaryon and see if this causes the disappearance of the antigens.

There is some evidence more directly linking ribosome biogenesis with mRNA transport. Girard et al (1965) showed that the small ribosome subunit in HeLa cytoplasm probably has a precursor in the form of a 45S RNP particle containing the 16S RNA. Henshaw et al (1965) found that a similar particle in rat liver cytoplasm incorporated radioactive RNA precursors into 16S RNA, when whole cells are pulsed and these components are extracted. The rate of incorporation was comparable with that of polysomal and transfer RNA. Poly-U (synthetic mRNA) preferentially binds to this particle. The 18S RNA from this 45S particle is able to stimulate amino acid incorporation into proteins in an E.coli cell-free system. In HeLa cells again, McConkey & Hopkins (1965) found that 3H-uridine appeared in the 45S particle before it did in the mature ribosomes, emphasising the probable precursor-product relationship, and that the 18S rapid label RNA from the 45S particle hybridized more efficiently to DNA than did rRNA as would be expected for mRNA.

As label first appears in the cytoplasm in this particle it is possible that the rRNA is already attached to the alleged mRNA on exit from the nucleus. These theories are in conflict with those of Henshaw (1968) and Samarina et al (1968) whose 30S particles were distinct in buoyant density and labelling characteristics from ribosomal precursor particles in rat liver.

The work to be presented shows that the blobs of anucleolar Xenopus appear to take in some nucleoplasmic RNA and this may represent the processing of HnRNA, although this specific possibility has not been tested. The fibrous region of the nucleolus in wild-type Xenopus behaves similarly. This comparison is supported by ultrastructural observations, as follows.

(iii) Comparison of the pars fibrosa and the anucleolar blob. The theme of this section is that the blobs in the nuclei of anucleolar Xenopus are identical in ultrastructure to the pars fibrosa (fibrous region) of the wild type nucleolus. The evidence is taken from Jones (1965 and unpub.) and Hay and Gurdon (1967) in which electron-microscopic observations were made on anucleolar nuclei and on wild type nuclei before and after gastrulation, and on these organelles

after chemical treatments.

The wild-type post-grastrula nucleolus consists of a central pars fibrosa of irregular folded shape consisting of an electron dense, fairly amorphous material which sometimes appears finely granular (smaller and less well defined granules than in the pars granulosa, mentioned next). Sometimes fine threads 40-100Å in diameter are discerned.

More or less surrounding and engulfing the pars fibrosa is the pars granulosa consisting of an amorphous matrix, denser than the surrounding chromatin but not so dense as the pars fibrosa, in which are embedded closely stacked granules 150-300Å in diameter.

The third region in the wild-type nucleolus is an amorphous much less electron dense region which may be DNP chromatin, visible in small pockets and channels throughout the nucleolus.

Blobs are more numerous and smaller than nucleoli. They are electron dense, sharply defined and often almost spherical. The matrix can sometimes be seen to be composed of densely packed 40-100Å diameter threads, although rarely granular caps are seen on the periphery. The cytochemistry of blobs is discussed later in this introduction.

Pregastrula wild-type nucleoli are more numerous and smaller than mature nucleoli. They are almost spherical, electron dense and are composed of 50Å diameter filaments. No granules are present. Around gastrulation a granular periphery develops, composed of 100-200Å diameter granules. At the same time these embryos begin to synthesize ribosomal RNA (Brown & Littna 1964). This and autoradiographic and cell fractionation studies discussed earlier in this chapter implicate the nucleolus as the site of rRNA synthesis and in particular the nucleolar granules as precursors of cytoplasmic ribosomes which are 200Å in diameter.

It is concluded that blobs are in many respects homologous with the pars fibrosa of the wild-type nucleolus and with pregastrula nucleoli. This view is supported by observations on AMD treated and acid extracted tissues (Jones unpub.). When tissues are extracted with HCl pH2 before fixation, a procedure considered to remove most of the histone proteins, the chromatin forms large convoluted threads which surround and extend from both the normal nucleolus and the blob. The nucleoli and blobs themselves both appear as masses of finer convoluted threads, differing only in size of the mass of threads. AMD does not markedly affect blobs but usually nucleoli were

changed into masses of fibrous material identical to that making up the blobs. Nucleolar segregation (Schoefl 1964) was occasionally observed.

To conclude this section; nucleoli synthesize rRNA, probably in the granular region. Evidence for the fibrous region being involved is not conclusive. Nucleoli are also involved in other functions. The possibility of their being involved in HnRNA and mRNA processing is discussed. Experiments with anucleolar Xenopus, which contain fibrous regions (blobs) in the nuclei, but no granular regions, are performed to try to resolve these points.

B: RNA Metabolism in Anucleolar Xenopus

Some aspects of anucleolar Xenopus have been discussed in the General Introduction (Chapter 1), and in Section A iii above. To summarize: in the homozygous anucleolar embryo there are not detectable ribosomal cistrons in the genome, no detectable rRNA synthesis and the nucleooli are replaced by fibrous blobs. The animal presumably uses maternally synthesized ribosomes carried over in the egg cytoplasm for protein synthesis. Earlier in this chapter (3.1 A iii) blobs are compared morphologically to the fibrous part of the wild type nucleolus.

Brown & Gurdon (1966) studied the size distribution and stability of RNA synthesized in 0-nu whole embryos. Embryos were labelled with radioactive RNA precursors, total RNA extracted from the whole embryos and analyzed by sucrose density gradient centrifugation. In all pulse and age conditions studied they found that the embryos synthesized 4S transfer RNA and heterodisperse RNA heavier than and well separated from the 4S RNA. The heterodisperse RNA was found to be DNA-like in base composition, emphasising that it is not rRNA. At all stages of development about 70% of RNA synthesis is into heavy (> 20S) heterodisperse RNA. This accumulates in the embryos up to the tailbud stage. After this stage a chase in unlabelled medium results in a degradation of the heavy RNA into light RNA which sediments at 10-20S. It seems very likely, because of the low pool size of the isotope used, that the heavy RNA is the precursor of the light RNA. This light RNA is as stable as 4SRNA (which does not degrade during the time course of these experiments.) The small amount of labelled RNA extracted from the ribosome fraction contained in addition to 4S RNA, light RNA only. Further attempts to find the cellular locations of heavy and light RNA failed because of degradation during separation. Important results to bear in mind when interpreting autoradiograms are that during a 2 hour pulse most of the RNA synthesized

is heavy (> 20S) and the total DNA-like RNA(dRNA) to 4S RNA ratio is about 4. When chased heavy RNA degrades and some of it forms stable light RNA such that the dRNA:4SRNA drops to about 0.5 after 5 hours, 0.3 after 10 hours and 0.2 after at least 94 hours.

It seems justified to call 4S RNA transfer RNA because Brown & Littna (1966) showed that there was no detectable 5S RNA synthesized in wild type Xenopus embryos until the swimming tadpole stage. Not surprisingly O-nu embryos, which die at this stage, were found never to synthesize 5S RNA. All of the RNA in these regions of the gradients then is transfer RNA.

Later Gurdon & Ford (1967) extracted RNA from polysomes separated from 3H-uridine labelled O-nu embryos and showed it to form a peak of radioactivity between the optical density peaks of 28S and 18S carrier rRNA.

Wallace (1962) labelled O-nu embryos with 3H-uridine and treated them for LMARG. He showed that O-nu nuclei label to about the same extent as wild type nucleoplasm but saw no particular concentrations of silver grains in the nucleus which might have represented blob labelling, (the blobs were not visible in the autoradiograms). The same result was obtained when methyl- 14C-methionine was used in the presence of puromycin in order to show

the sites of RNA methylation.

0-nu gave uniform labelling over nucleus and cytoplasm. He also showed (Wallace 1962) cytologically that blobs contain RNA, arginine (presumably in the form of an arginine-rich protein) and alkaline phosphatase. DNA was detected but was thought to be entirely in the associated chromatin. Gurdon & Hay (1966) also claim that blobs do not incorporate radioactive precursors into RNA.

Hallberg & Brown (1969) failed to find synthesis of any of 10 specifically assayed ribosomal proteins in 0-nu embryos and in pre-gastrula wild type embryos, which also do not have nucleoli. They concluded that the synthesis of ribosomal RNA and ribosomal proteins are coordinated.

Jones (1971) used EMARG to investigate RNA synthesis in 0-nu muscle tissue. The findings for the cytoplasm are discussed in the next section. He found that blobs do incorporate 3H-uridine sporadically (sometimes labelled and unlabelled blobs are seen in the same nucleus even after a 20 hour pulse) and often the incorporation is on the edge of the blob. The blob associated chromatin is active in RNA metabolism. The fibrous region of the nucleolus labelled

in 12 minutes but blobs were not significantly labelled until after 45 minutes. Blobs also incorporate 3H-leucine and so probably synthesize protein.

C: RNA metabolism in Muscle Cells

The greatest advance made in this field is the isolation of active myosin messenger RNA by Heywood's group at the University of Connecticut. They used a high ionic strength medium for extracting polysomes. Myosin precipitates at low ionic strength and the polysomes coprecipitate, reducing the yield. Use of the high ionic strength medium gives a good yield of polysomes from embryonic chick skeletal muscle, which were shown to be active by the amino acid incorporation profile following the polysome optical density profile on a sucrose density gradient. The largest group of polysomes was shown by EM to contain 50-60 ribosomes per polysome and its product, synthesized in vitro, electrophoresed in the same position as pure myosin (Heywood et al 1967). Other distinctly sedimenting groups of polysomes were found in vitro to synthesize actin (15-25 ribosomes per polysome), and tropomyosin (5-9 ribosomes) plus unknown substances (probably due to contamination of these polysomes with others of similar sizes) as identified by polyacrylamide gel electrophoresis and cyclic purification

to constant specific activity with pure carrier proteins (Heywood & Rich 1968). The RNA was extracted from the myosin synthesizing polysomes and shown to be still active by its ability to direct myosin synthesis on monosomes from the same preparation (Heywood & Nwagwu 1968). This RNA was then used to direct myosin synthesis on heterologous (chick reticulocyte) ribosomes (Heywood 1969), but requiring a factor present in a wash of muscle tissue ribosomes. The RNA was further characterized as predominantly 26S (Heywood & Nwagwu 1969). Its product was also further characterized as predominantly the heavy chain of myosin. This led to a search for polysomes synthesizing the light chain of myosin and it was found to be synthesized on the 4-9 ribosome class of polysomes also implicated in tropomyosin synthesis (Sarkar & Cooke 1970 and Low et al 1971). Independant synthesis of small and large subunits, the small apparently being synthesized faster than the large, was confirmed in a double label experiment in vivo by Brivio & Florini (1971).

The heavy and light chain myosin polysomes as well as the actin and tropomyosin polysomes were all calculated to be about the right size for containing a monocistronic message. Heywood & Thompson (1971) found that the 26S mRNA binds first to the small 40S ribosome subunit, which then, along with tRNA binds to the

60S large ribosome subunit to form a 75S ribosome complex. It is not know what further steps are involved in polysome formation. Both steps require unidentified factors present in high-salt washes of muscle monosomes.

In 1963 long polysomes with a helical configuration were first observed directly by EM in muscle tissue (Behnke 1963 and Waddington & Perry 1963). These contain in excess of 40 ribosomes and so are probably the heavy chain myosin synthesizing polysomes. This is supported by the findings of Allen & Torrence (1968) who extracted polysomes from embryonic chick muscle and separated them into long and short polysomes in a sucrose density gradient. The long ones were observed in EM to be about 60 ribosomes long and to bind antimyosin antibodies along half their lengths, showing them to contain nascent myosin. It was not possible in these whole mounts to discern if there was any helical arrangement of the polysomes. Nihei (1971) separated free and membrane-bound polysomes from rat muscle tissue and found that the free polysomes synthesized much more myosin than the bound polysomes.

RNA metabolism has been studied in muscle by several workers using LMARG. Sirlin & Elsdale (1959) studied adenine and methionine uptake into RNA and protein

respectively in the embryonic myoblasts of wild type Xenopus and Bufo, concentrating their attention mainly on nuclear structures. They found the highest uptake of both compounds in the region of the nuclear membrane and the lowest in the chromatin. The nucleolus, including associated chromatin, was the lowest. Chase experiments were not done and so precursor-product relationships could not easily be inferred from their data. Similar results had been obtained with various embryonic cell types. Marchok (1966) found that mononuclear myoblasts in 11-14 day chick embryos incorporated 3H-uridine and 3H-cytidine into RNA in the nucleus and cytoplasm but multinucleate myotubes did not. At 18 days, however, the nucleus and cytoplasm of myotubes did take up label. She concluded that myoblasts synthesize RNA and then stop at fusion, then resume synthesis some days after fusion. In 1970 Marchok showed that developing muscle showed a decline in RNA polymerase activity, whether free or chromatin bound, active on native or denatured DNA, or magnesium or manganese activated (i.e. preferring rDNA or non-rDNA respectively). This was found not to be due to build up of inhibitors as the extract was responsive to exogenous E.coli enzyme. The same rise in RNA synthesis rate followed by a decline at fusion, paralleling myosin synthesis rates, was found by biochemical procedures by Coleman & Coleman (1968) in chick muscle tissue

culture, FUdR being used to selectively kill mononuclear cells for observation of fused myotubes. When AMD was applied to block RNA synthesis both newly formed and mature myotubes were morphologically devastated within 24 hours. A different response to AMD was found by Yaffe and Feldman (1964) who found that a concentration of AMD which was sufficient to block all autoradiographically detectable RNA synthesis killed mononuclear myoblasts within 24-48 hours whereas fused myotubes stayed healthy and contracting for 48-56 hours. This study was done on cultured rat muscle cells and supports the concept of stable mRNAs for myofibrillar proteins being synthesized some time before fusion. The concentration of AMD used had no autoradiographically detectable effect on protein synthesis in myotubes. That the mRNAs are not only stable but cease to be synthesized after fusion is made likely by the finding (Yaffe & Fuchs 1967) that when cultured muscle cells are given 20 minute pulses of 3H-uridine at various developmental stages and subjected to autoradiography, nuclei become labelled in mononuclear cells right up to the stage of fusion and then stop incorporating label.

Jones (1971) studied non-ribosomal RNA metabolism in 0-nu Xenopus embryo muscle cells. Nuclei appeared to become maximally labelled with 3H-uridine after 45

minutes. Great differences in intensity of labelling between different nuclei were often observed, probably denoting asynchrony of development. Also differences of intensity of labelling were observed in different regions of the same nucleus, the significance of which is not known, except where the regions of chromatin around blobs were heavily labelled. This was thought to perhaps represent a function of the blob in processing mRNA. The cytoplasm of muscle cells did not become appreciably labelled until after a 6 hour pulse although slight labelling was observed after a 12 minute pulse. When the cytoplasm was heavily labelled after a 10-20 hour pulse it was observed that the myofibrils had 4-6 times the specific activity of the interfibrillar cytoplasm, indicating that non-ribosomal RNA becomes strongly associated with existing myofibrils. That this RNA was probably involved in the translation step of protein synthesis was shown by a similar high uptake of 3H-leucine into myofibrils. Morkin (1970) observed the incorporation into protein (cycloheximide sensitive) of 3H-leucine into the edges of myofibrils by EMARG, having extracted soluble proteins from the pulse labelled rat diaphragm muscle with glycerol.

The theory that polysomes attach to existing myofibrils for the synthesis of at least some of the myofibrillar

proteins is supported by other observations than the autoradiographic observations mentioned above. Heuson-Stiennon (1964) observed in embryonic rat muscle long helical polysomes free in the cytoplasm but having attached to them filaments very similar in appearance to the myosin (thick) filaments of the myofibrils. Thus at least the association of myosin molecules to form a filament (filamentogenesis) appears to take place in association with the polysome in this case. Fischman (1970) argues that because purified myosin molecules can form thick filaments in vitro by adjusting the pH of the solution (Huxley 1963 and Kaminer & Bell 1966) then there is no need to postulate the involvement of polysomes in filamentogenesis. On the contrary, in the absence of the demonstration of these ionic conditions in the cell it seems necessary to postulate some other mechanism which explains the observations. Other observations include the association of long helical polysomes with myofibrils and with regions of the cell surface where myofibrils were seen to originate in rat heart cell cultures (Cedergren & Harary 1964), the association of ribosome clusters of up to 7 ribosomes with thin filaments and myofibrils in frog muscle spindles (Andersson-Cedergren and Karlsson 1967) and the association of ribosomes with the thick filaments of myofibrils in human

foetal and regenerating mouse muscle (Larson et al 1969 and Larson et al 1970). More discussion of the role of polysomes in filamentogenesis and fibrillogenesis is found in Chapter 2.1. The autoradiographic data presented here supports the theory that some myofibrillar proteins are synthesized and presumably polymerized into filaments in situ, in the fibril.

3.2. MATERIALS AND METHODS

Abbreviations:

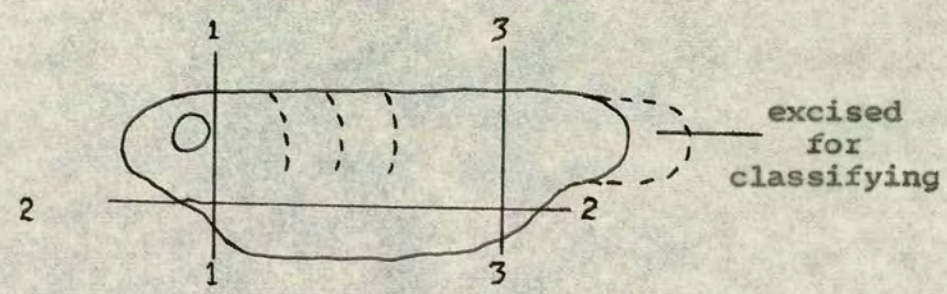
sp.ac.	specific radioactivity in silver grains per square micron of section.
WT	wild type (1 or 2 nucleoli).
0-nu	anucleolar mutant.
3HU	uridine-5'-tritium.
10+30	10 minutes pulse of 3HU followed by 30 minutes chase in unlabelled medium. Figures represent minutes unless stated.
N	nucleoplasm (not counting blobs or nucleoli).
PF	pars fibrosa of the nucleolus
PG	pars granulosa of the nucleolus
B	blob
M	myofibrils
NMS	non myofibrillar sarcoplasm
C	cytoplasm of unclassified cells.

Xenopus Embryos:

These were obtained by crossing Xenopus laevis adult toads heterozygous for the anucleolar condition (Chapter 1) maintained as a breeding colony at this laboratory. Males and females were each injected subcutaneously with 0.3 ml of a solution of 1500 IU of chorionic gonadotrophin (Pregnyl, Organon labs. Ltd., Morden) in 2 ml distilled water. 8 hours later the

females were injected with a further 0.4 ml of the solution. Fertile eggs were collected the next morning and stored in water with an air bubbler at room temperature (RT), i.e. about 20°, or at 18° to slow down development so that there was plenty of time to classify the embryos. Males in poor breeding condition (no black pads on the fore-limbs) were occasionally subcutaneously injected with a 0.21 ml of solution of 1000 IU serum gonadotrophin (Gestyl, Organon) in 3.4 ml of distilled water 1 week prior to mating. Embryos were staged according to Rugh (1948), not according to the Niewkoop & Faber tables. Some hours before the stage required the jelly and membranes were removed from a batch of embryos with hypodermic needles and finely ground watchmakers' forceps. It is best to do this, where possible, after the muscular response stage as the membranes are not so close to the embryo after this stage. Naked embryos are then transferred to sterile Barths+ medium (a physiological non-nutrient saline solution made up from Oxoid phosphate buffered saline tablets and Oxoid mineral salt tablets according to the makers.) They were washed several times in this solution to remove bacteria and pieces of membrane and stored at RT in this solution until the stage required, (usually between muscular response and hatching). The embryos were then screened for anucleolar homozygotes (0-nu)

by removing a small portion of the tail with finely pointed tungsten wires (made by cautiously dipping the end of a mounted tungsten wire in molton sodium nitrite) and gently squashing the pieces between a microscope slide and a coverslip such that the cells were disrupted but the nuclei were not flattened. Phase contrast microscopy at x400-x800 revealed the presence or absence of nucleoli. As WT embryos do not develop true nucleoli until the gastrula stage screening of O-nus cannot be done before this stage. After hatching the O-nu blobs enlarge and become difficult to distinguish from nucleoli. Screening is ideally done when classification is easy, at or around tailbud stage, giving plenty of time before death for experiments. As a rough check on the accuracy of typing of the embryos the ratio of O-nu to total embryos should be approximately 1:4. The absolute check on accuracy is the electron-microscopy. The somite regions of WT and O-nu embryos were then dissected out with tungsten wires. The diagram below illustrates the region excised. The numbers refer to the order of the cuts.



The somite tissue was then cut transversely into 3 or 4 pieces. The pieces contained somitic muscle, ectoderm and notochord. It is however possible to isolate the somitic tissue if a little trypsin solution is added to the Barths for a few minutes. Somite pieces were used immediately in labelling experiments.

Labelling:

Somite pieces from stage 19-20 embryos (WT and O-nu) were pulse chased with tritiated uridine (3HU) for 10 minutes ± a 30 minute chase (coded 10+0 and 10+30 respectively) (WT only), 30 minutes ± a 30 minute chase (coded 30+0 and 30+30), and 60 minutes ± a 6 hour chase (60+0 and 60+6) and somite pieces from embryos of various stages from 10-18 were labelled for 15 hours (the developmental series). The results described here are mainly the pulse-chase experiment plus stage 18 of the developmental series, some of the rest of which are used in Chapter 2. The somite pieces, in Barths+ were washed in MEM (for the developmental series, MEM= 1x Eagles minimal essential medium, Hanks based + non-essentia amino acids (Wellcome Laboratories) plus 10% foetal bovine serum (Flow Laboratories), plus 40µg/ml streptomycin sulphate (Glaxo), plus 100µg/ml, benzyl penicillin (Glaxo), plus 1.1% sodium bicarbonate: for the pulse-chase experiment, the same medium was used except sodium bicarbonate was omitted and the foetal bovine

serum was replaced with 0.1% BSA (Bovine Albumen Powder, Fraction V from bovine plasma, Armour Pharmaceutical Co.Ltd.). After MEM washing the embryo pieces were transferred to MEM containing 50μCi/ml 5'-tritiated uridine (1mCi/ml, 17.25Ci/mM, Radiochemical Centre, Amersham), up to 20 somite pieces per 0.5ml, in glass tubes closed with rubber bungs in a 29° incubator. After pulsing, somite pieces were withdrawn from labelled medium and washed three times in fresh MEM, then either fixed or transferred to a fourth batch of fresh MEM for chasing, and then fixed. Some somite pieces were pulsed for 16 hours in labelled medium ± 1μg/ml. AMD (Dactinomycin, Lysogen Cosmovac, Merck, Sharpe & Dohme) and others were preincubated for 1 hour in unlabelled MEM containing 1μg/ml. AMD, washed in MEM and then incubated in labelled MEM for 15 hours.

Glutaraldehyde Fixation:

Some 15 hour pulsed, MEM washed somite pieces were fixed for 30 minutes in glutaraldehyde fixative and washed in more than 6 changes of phosphate buffer (see below) for more than 4 hours, then RNAsed, then phosphate buffer washing was repeated before osmic acid fixation. All of the rest of the pulsed, chased and washed somite pieces were fixed overnight in 6.25% filtered glutaraldehyde (Taab) in 0.1M

phosphate buffer pH7 (3 volumes of 13.6g/L KH_2PO_4 + 8 volumes of 17.4 g/L K_2HPO_4). Unincorporated radioactivity is then removed (Jacob 1971) by washing for 2-3 days at 4° in 0.1M phosphate buffer changed twice daily.

RNAse & TCA:

Some 15 hour pulsed, 30 minute fixed and washed WT somite pieces were washed three times in 5% TCA (trichloroacetic acid) for 10 minutes each at 0° to test if the phosphate buffer washing removes all of the non-incorporated label. Other pieces were incubated for 1 and 6 hours at 37° in 10 and 100µg/ml RNAse in 0.1M phosphate buffer pH7 (RNAse A, 5x crystallized from bovine pancreas, Sigma) or in buffer without RNAse. All pieces were then washed for more than 4 hours in more than 6 changes of phosphate buffer at 4°.

Osmic acid fixation and araldite embedding:

The glutaraldehyde fixed and phosphate washed somite pieces were then fixed for 2 hours at 0° in a solution of osmic acid (= osmium tetroxide) from a mixture of 5 ml of 2% aqueous osmic acid (Gurr), 1 ml of 0.01% aqueous magnesium acetate, 2ml. deionized water, 0.291g sucrose and 1 ml. Sorenses phosphate buffer (0.87ml. of 12g/L Na_2HPO_4 + 0.13ml. of 9g/L KH_2PO_4),

rinsed 2x1 minute at 4° in 0.1M phosphate buffer pH7, then at RT washed 3x5 minutes in distilled water, and dehydrated in ethanol solutions (70% 15 minutes, 95% 15 minutes, and 100% 3x30 minutes) and in propylene oxide (= epoxy-propane) for 4x15 minutes in 50% propylene oxide in ethanol and 30 minutes in 100% propylene oxide. Embedding medium is 10 ml. araldite epoxy resin, 10 ml. hardener, 0.44 ml. accelerator (all Ciba) and 1 ml. di-n-butyl phthalate. Somite pieces were transferred from tubes individually to gelatin pill capsules (Parke-Davis) in propylene oxide and the liquid exchanged for 50% v/v propylene oxide in embedding medium for 30 minutes and then 100% embedding medium. The capsules were baked at 65° for 2-3 days.

Sectioning:

Thick (2μ) and thin (gold interference colour) sections were cut for LMARG and EMARG respectively on a Sorvell Porter-Blum MT-1 hand ultratome with glass blades. Thick sections were checked for location and orientation of myofibrils before final trimming and thin sectioning. Thick sections were transferred with a needle onto drops of water on gelatinized microscope slides (slides soaked in chromic acid for 1 day and running tap water for 2 days, rinsed in distilled water and ethanol and dried, then dipped in a solution

of 0.5% gelatin + 0.1% chromium aluminium sulphate dissolved at 90° and filtered, for 20-30 seconds and dried at 37°). When the drops of water had evaporated the slides were gently heated on a gas flame to stick on the sections ready for LMARG.

Thin sections were picked up from the ultratome water trough on nickel EM grids, 100-200 grids/inch (Mason & Morton Ltd.) which had been coated with formvar and carbon in the following way. Clean, dry glass microscope slides were wiped with a dust free tissue and, (if possible) on a dry day dipped into a 0.5% bubble free solution of formvar (Taab) in chloroform and air dried. The edges of the slide were scratched with a scalpel blade and the film of formvar floated onto a dust free water surface by gently lowering the slide below the surface at an angle. A film with a deep gold to purple interference colour was found to be compatible with the rigidity and translucency requirements of EMARG. The grids were floated on the film and then picked up on newspaper (unprinted). The newspaper was dried and placed in a vacuum carbon evaporator (AEI) and coated with a layer of carbon sufficiently thick to make a piece of filter paper appear pale chocolate brown. After picking up the sections on these grids surplus water was carefully removed with filter paper and the grids allowed to air

dry. They were then stained by floating the grids individually on drops of 2% aqueous filtered uranyl acetate for 15 minutes at RT, washed twice in distilled water and then floated on drops of Reynolds lead citrate stain for 10 minutes (1.3g lead nitrate, 1.76g sodium citrate $2H_2O$, 30 ml deionized water, shaken for 30 minutes, then 8ml 1N sodium hydroxide (carbonate free, BDH) added and made up to 50ml with deionized water then filtered before use), in a dish containing sodium hydroxide pellets to remove atmospheric CO_2. The grids were rinsed in 0.1N sodium hydroxide (carbonate free) and in 2 changes of distilled water and then dried on filter paper. The grids bearing the stained sections were then coated with carbon for a second time, this time with a thinner layer so as to form the faintest detectable negative shadow on a piece of underlying filter paper. This prevents the stain from interacting with the photographic emulsion to be applied and also obviates the need to stain the developed ARGs with uranyl acetate, which stains the gelatin of the emulsion (Jacob 1971).

Autoradiography:

Slides bearing thick sections were coated in photographic emulsion by dipping, under an amber safelight, in K2 Nuclear Research Emulsion (Ilford) melted with

an equal volume of distilled water at 45° for 10 minutes. They were fan dried at 20° at 70%RH and then stored at 4° in foil wrapped black plastic boxes for the stated exposure times (2-10 weeks). They were then developed in D19b (Kodak) for 4½ minutes at 20°, washed twice in distilled water, then fixed in 16% aqueous Fix-Sol (Johnson) for 8-10 minutes at 20°, rinsed in distilled water for 30 minutes and air dried. Slides were stained, usually in 0.2% aqueous toluidine blue (Gurr) rinsed in distilled water, air dried and mounted in immersion oil under a coverslip. LMARGs are used elsewhere in this thesis but for the work in this chapter they were only used for RNAse, TCA and AMD controls and to check the pulse chase material for incorporation before thin sectioning.

Grids were stuck 5-6 at a time by the edges onto double sided sticky tape which was stuck to the long edges of rectangles of glass about 5x15mm. The glass mounted grids were then placed individually on a small table about 4cm high with a top smaller than the mount, in a dark room illuminated by an amber safelight at 20° and 70%RH. 5g of L4 Nuclear Research Emulsion (Ilford) were melted with 10ml distilled water for 10 minutes at 45°. The emulsion was then poured into a small petri dish (glass) which was placed on ice for 30 seconds and stood at RT until the next part of the

procedure worked (about 15 minutes). An expanding loop, made by passing doubled piano wire through a 1ml plastic syringe minus plunger with the loop at the nozzle end and fixing one free end to the plunger end of the syringe so that pushing the other free end of the wire into the syringe expands the loop, was adjusted to about 1cm diameter (the elastic limit of the wire) and dipped into the partly gelled emulsion, which should be cool enough to have about the same consistency as cold custard. The loop was withdrawn and rapidly expanded to about 5cm diameter upon which faintly discernible blue and purple interference colours should stop swirling within seconds. When swirling stops the gel is stable (uniform packing of crystals) and at this stage the loop was held parallel to the grid surfaces and brought down over them, fairly rapidly, such that the mounted grids passed through the loop, bursting the film. Careful observation of the glass mount revealed a wave of drying passing over the attached film. The interference colour of the dry film was uniform gold indicating a uniform close packed monolayer of crystals. It was found to be provident to check this on a clean glass slide before starting and at 10 minute intervals throughout coating as the emulsion may get too thick and have to be remelted and pregelled. This technique is essentially described in Jacob (1971). Mounted

grids coated in this way were stuck to glass slides by the glass mount with double sided sticky tape and the slides stored in black plastic slide boxes with dehydrated silica gel bags (water vapour reduces emulsion sensitivity) wrapped with foil at 4° for the stated exposure time, usually 12-18 weeks. Grids were developed, attached to the mounts and slides, in D19b (Kodak) for 2½ minutes at RT, carefully washed twice in distilled water and rinsed for 5 minutes at RT in F24 (Kodak), freshly made and rinsed in several changes of distilled water for 1 hour. They were then detached from the mounts, thoroughly washed in distilled water and restained while still wet in Reynolds lead citrate (see above) for 10 minutes, washed in sodium hydroxide and water and air dried.

EM autoradiograms were viewed on an AEIEM6G electron microscope at 60kV with an objective aperture of 50 microns. Photographs were taken on roll film mainly at magnifications of 2.5-16K diameters.

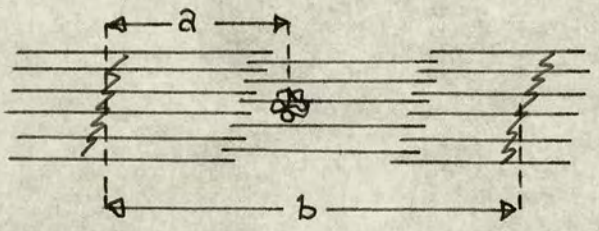
Estimation of specific activities:

Specific activities were measured as silver grains per square micron from the photographs. Each photograph was traced on paper tested for fairly uniform density (YD Cut Bank) demarcating nuclei, blobs, pars fibrosa, pars granulosa, myofibrils, non-myofibrillar sarcoplasm

in cells classified as muscle cells (containing myofibrils) or unclassified (non-muscle or very early myoblast) cells. Sample tracings are shown in the results section (Plates 32 and 33). Silver grains in each area were counted (overlapping grains were scored as $\frac{1}{2}$ in each area) and recorded separately for each cell type for each photograph. The weight of each tracing paper was recorded. The separate areas were cut out and weighed, and the weights recorded. In a given experiment the specific activity in grains per square micron of a given structure is the total number of silver grains observed in the structure divided by the total area in square microns observed, calculated from the weight of paper, the magnifications of the individual photographs and the densitites of the individual sheets of tracing paper. The standard error of this mean is calculated as shown in appendix 7.3 taking each photograph as a unit observation for that structure, using an equation provided by Mr Shukla (Statistics Department) and a computer program provided by Cathy Paver (Genetics Department) both of Edinburgh University.

In another analysis the distribution of radioactivity along sarcomeres was measured. To do this the centres of grains lying over myofibrils were located accurately by measurement of the centre of the diameter of an

imaginary circle just large enough to enclose the silver grain. Then the distances from the centres of the grains to the nearest Z band were measured in a direction parallel to the myofilaments. Lastly the inter-Z distance through the grain centre and along the myofilaments was measured. The ratio of the two measurements x100 is the grain position:-



$\frac{a}{b} \times 100$ = Grain position

Thus Z=O and M=50. The % of total myofibrillar grains observed in an experiment occuring in 10% strips along the sarcomere is plotted against sarcomere distance. This method is useful in combining data from many myofibrils in different states of contraction.

3.3. RESULTS

The results are displayed on the following micrographs, graphs and histograms. The micrographs, being only a small selection, do not show a great deal in themselves. Even fairly large differences in grain density between two experiments in a cell structure displayed on a histogram compiled from many micrographs may not be discernible by comparing a single pair of micrographs. Full results and computations are in the appendix (7.3)

Micrographs:-

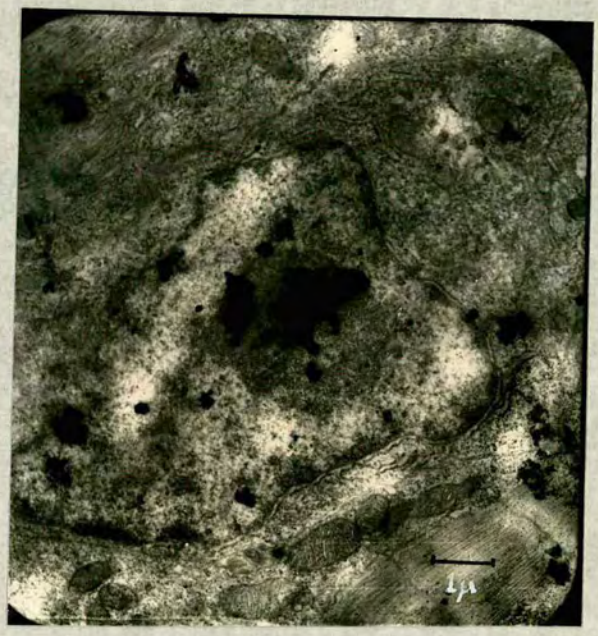


PLATE (1)

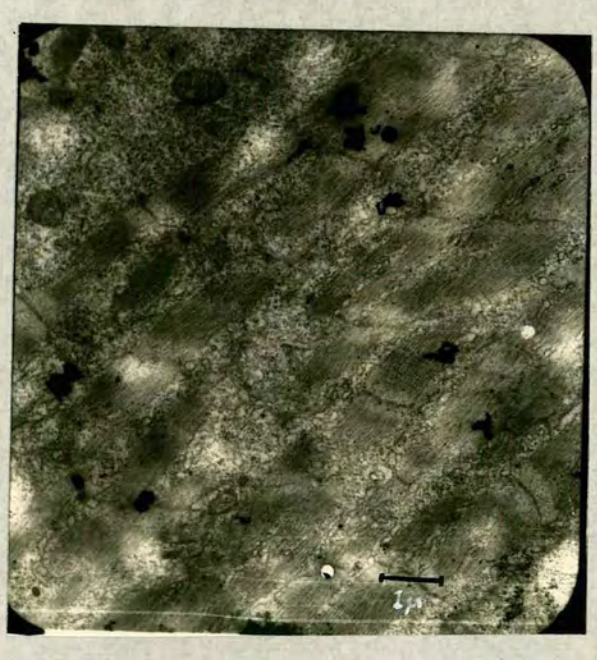


PLATE (2). WILD-TYPE. 10 minute pulse.

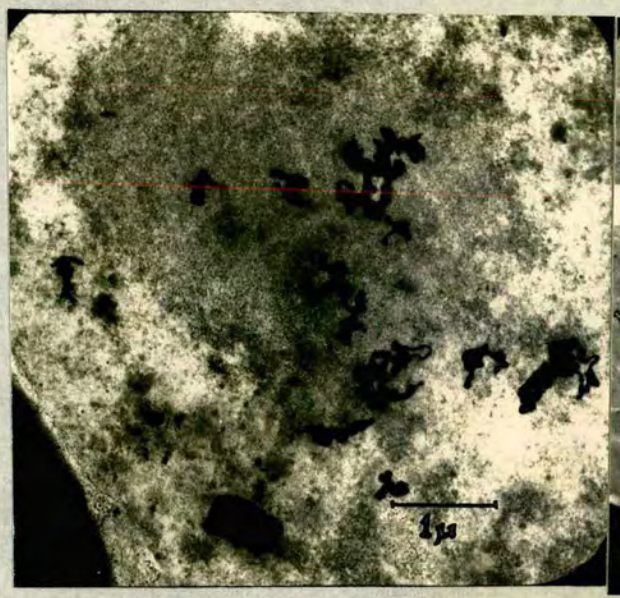


PLATE (3)

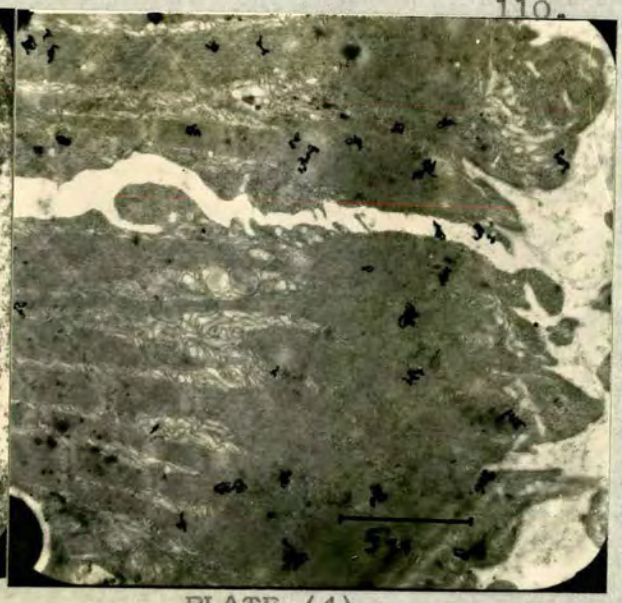


PLATE (4)

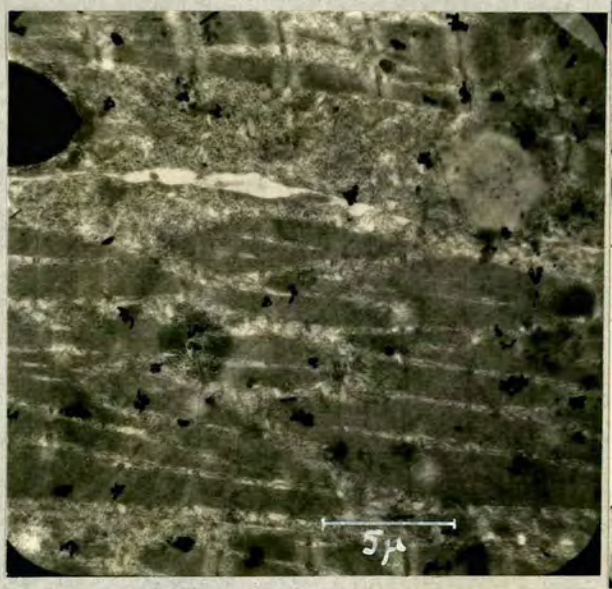


PLATE (5)

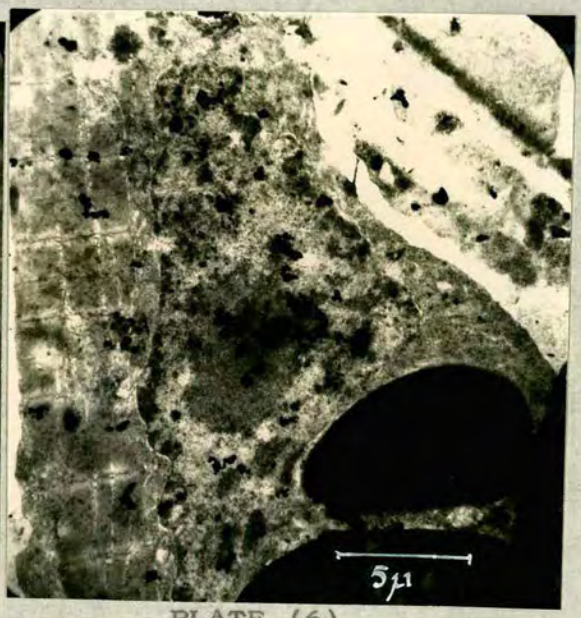


PLATE (6)

WILD-TYPE. 10 minutes pulse, 30 minutes chase.

PLATE (7). O-NU. 10 minutes pulse

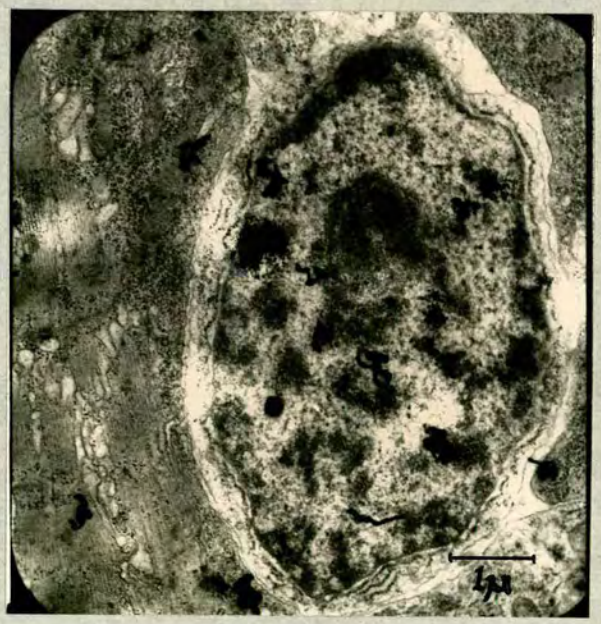


PLATE (8)

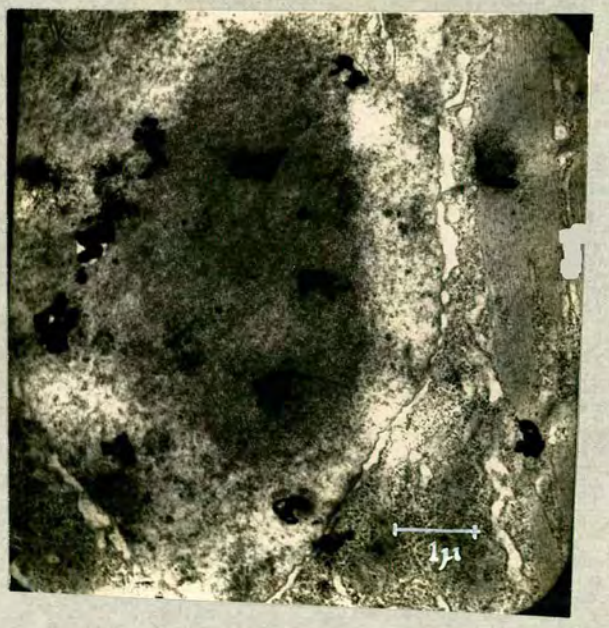


PLATE (9)

WILD-TYPE. 30 minutes pulse

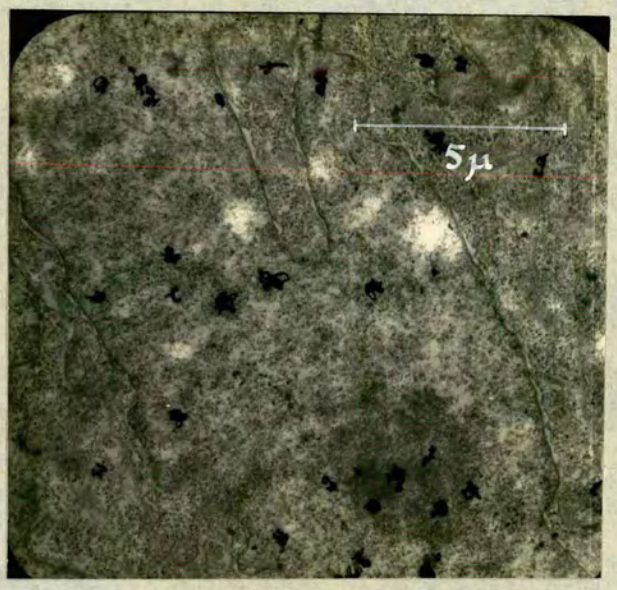


PLATE (10)

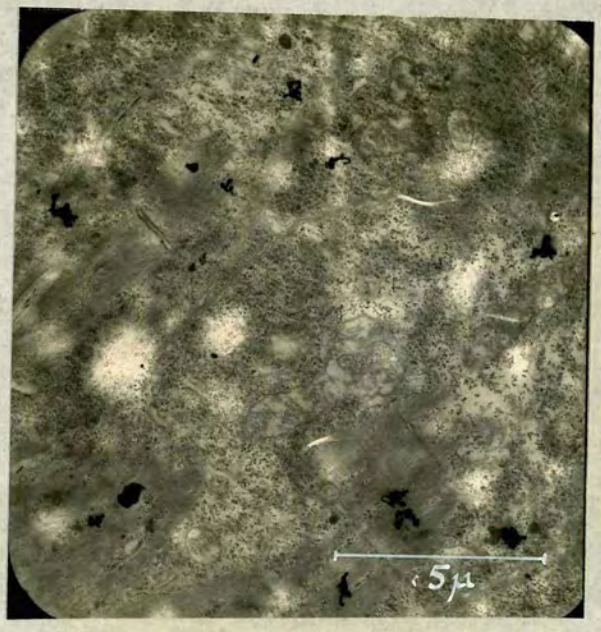


PLATE (11)

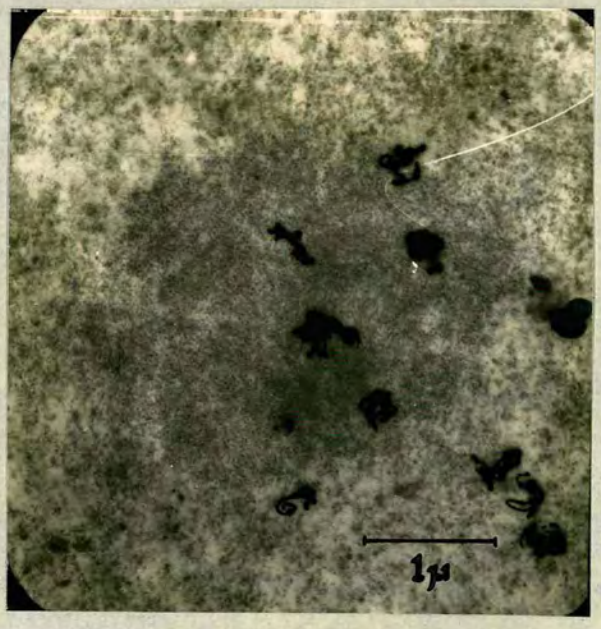


PLATE (12)

WILD-TYPE. 30 minutes pulse + 30 minutes chase

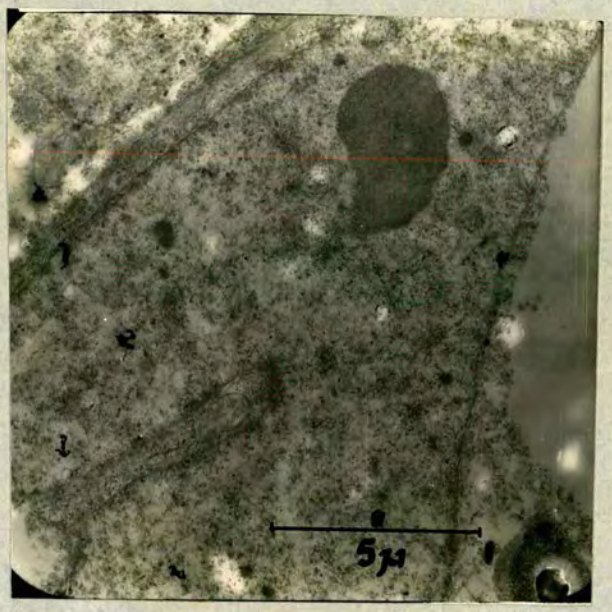


PLATE (13)

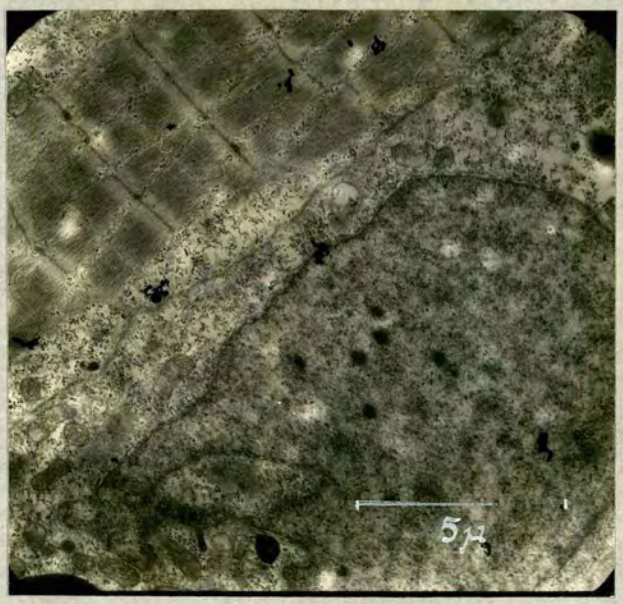


PLATE (14)

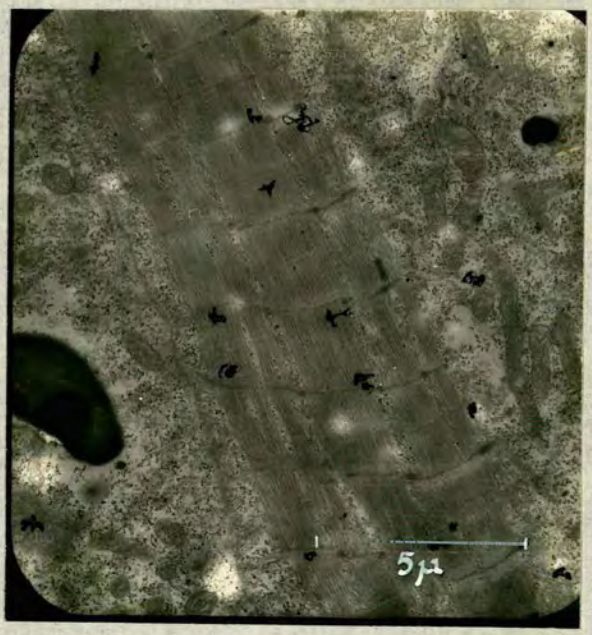


PLATE (15)

O-NU. 30 minutes pulse

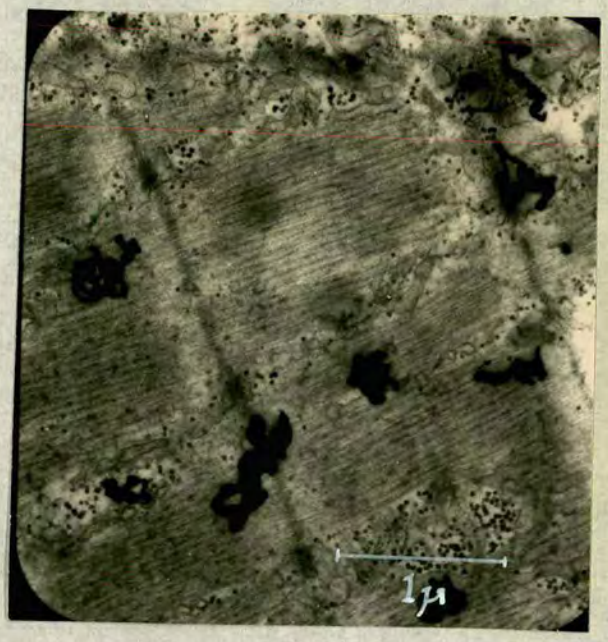


PLATE (16)

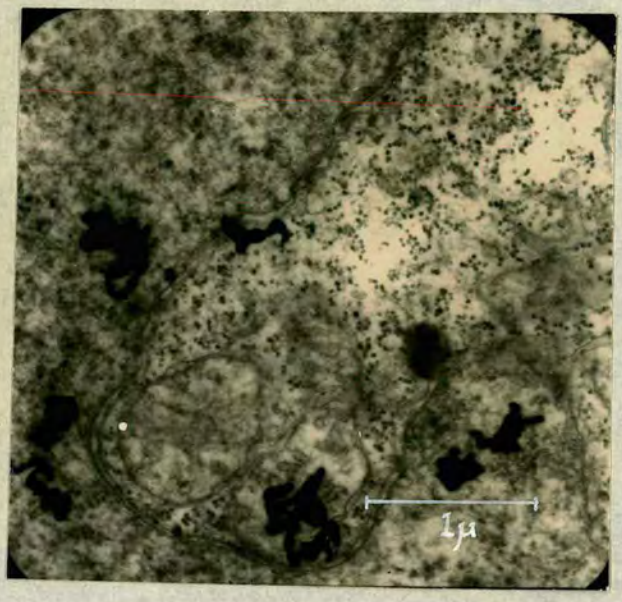


PLATE (17)

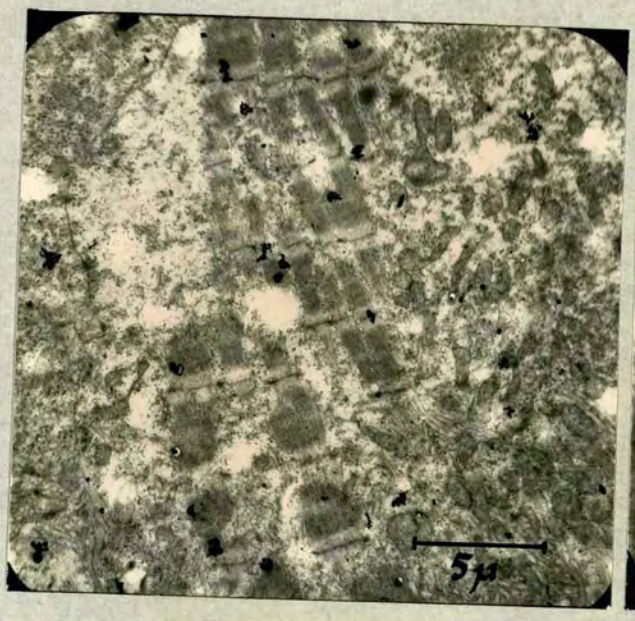


PLATE (18)

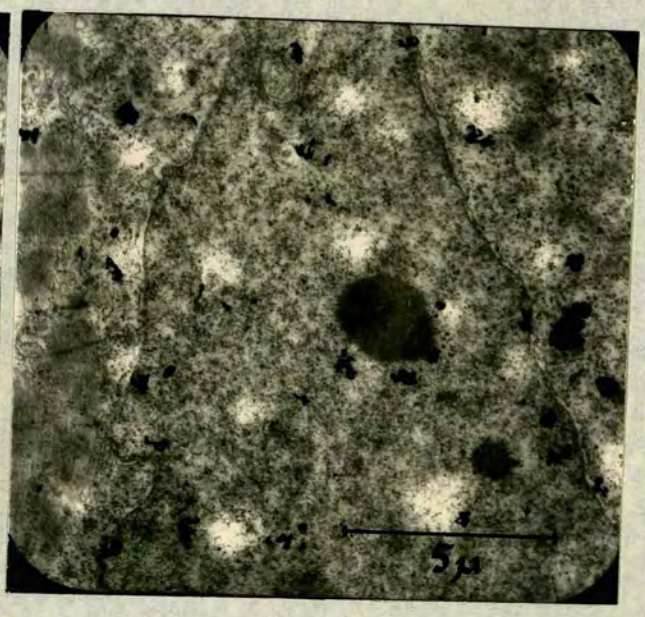
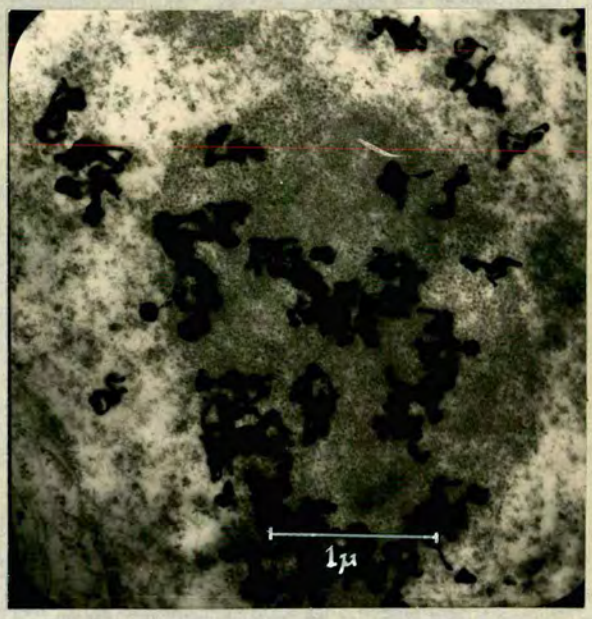


PLATE (19)

O-NU. 30 minutes pulse + 30 minutes chase



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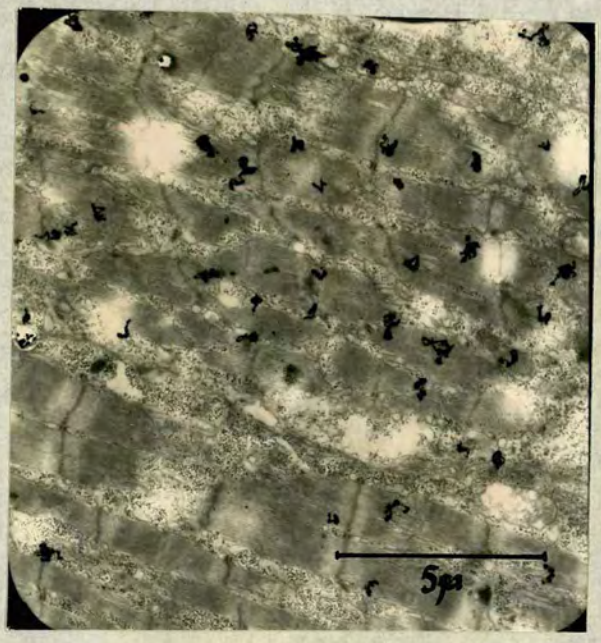

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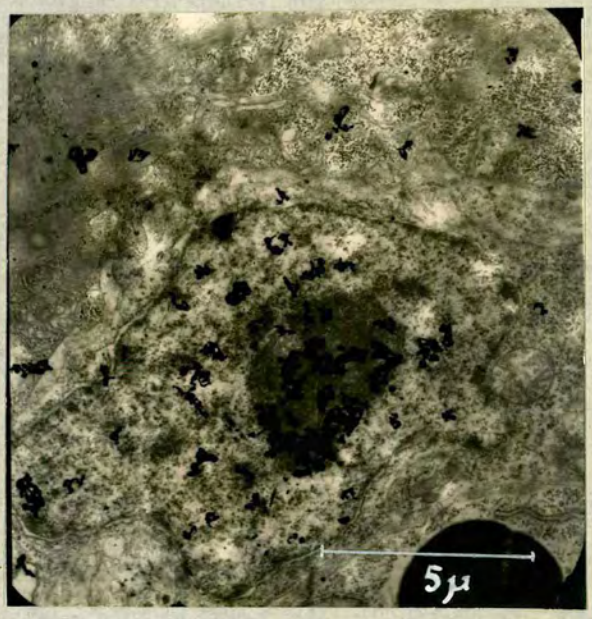


PLATE (22)

WILD-TYPE 60 minutes pulse

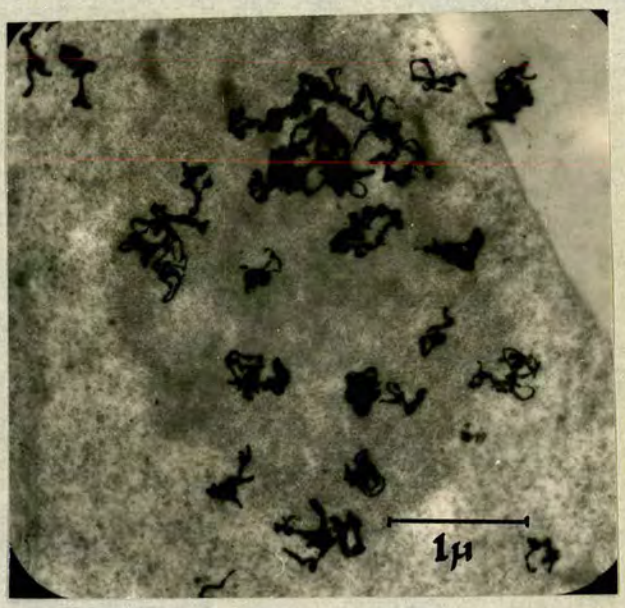


PLATE (23)

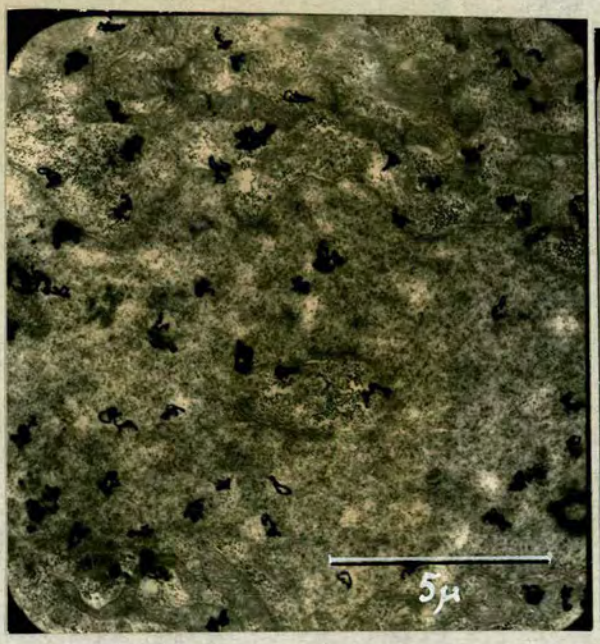


PLATE (24)

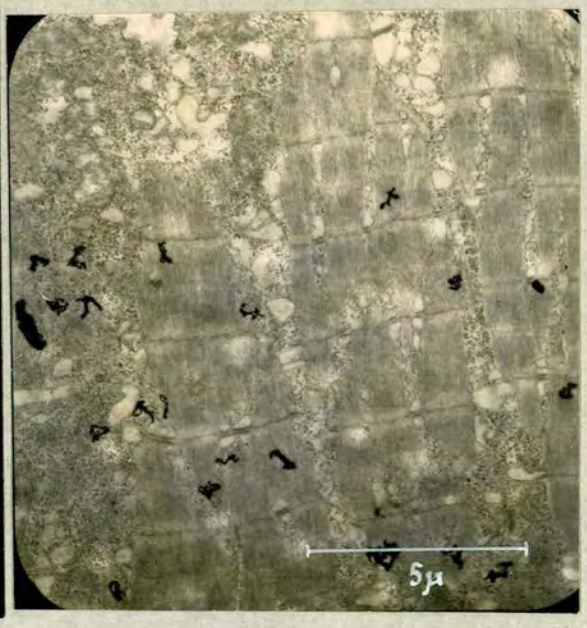


PLATE (25)

WILD-TYPE. 60 minutes pulse + 6 hour chase

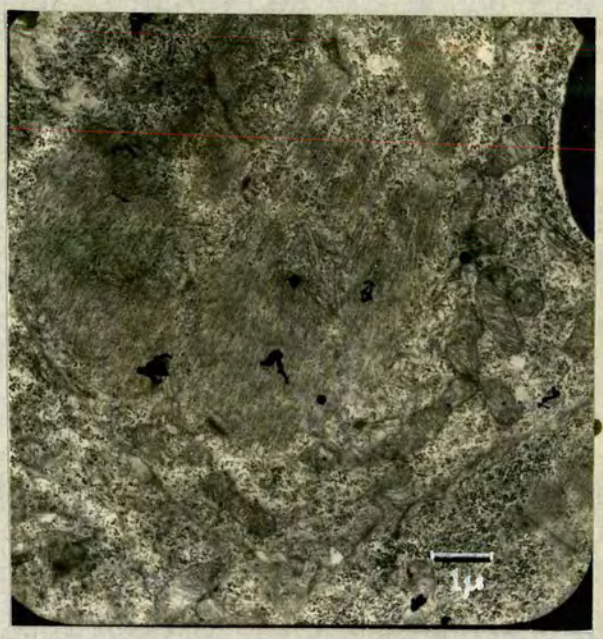
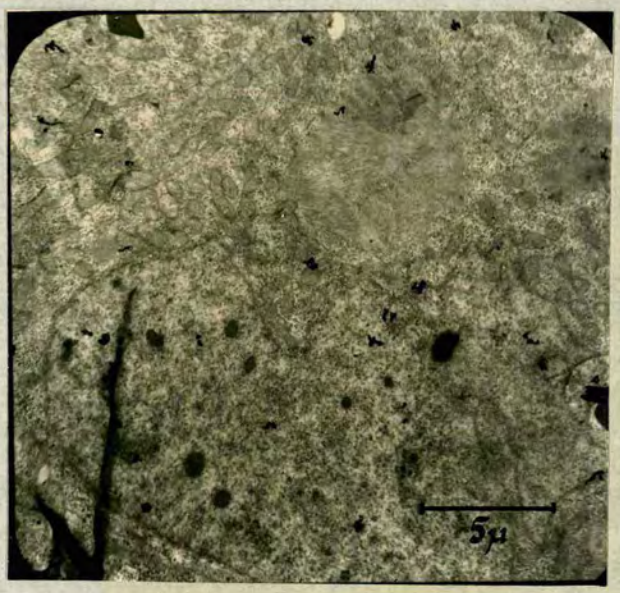


PLATE (26)



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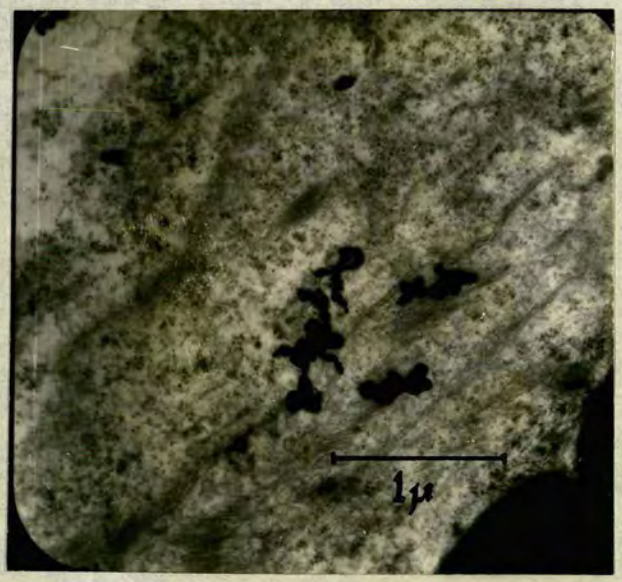


PLATE (28)

O-NU. 60 minute pulse

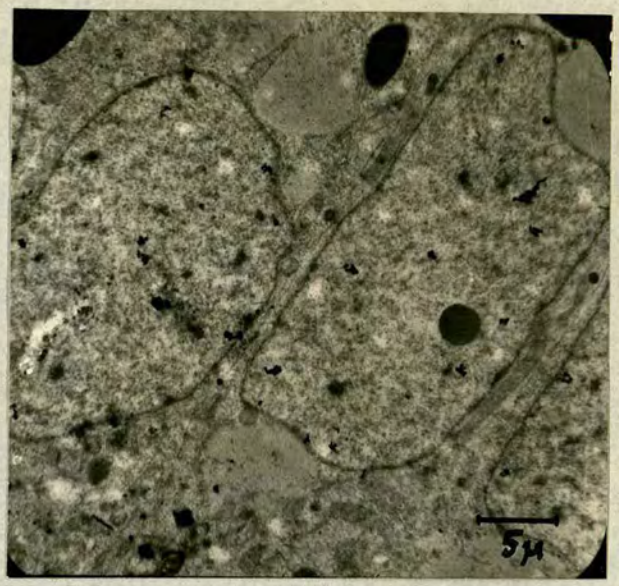


PLATE (29)

PLATE (30)

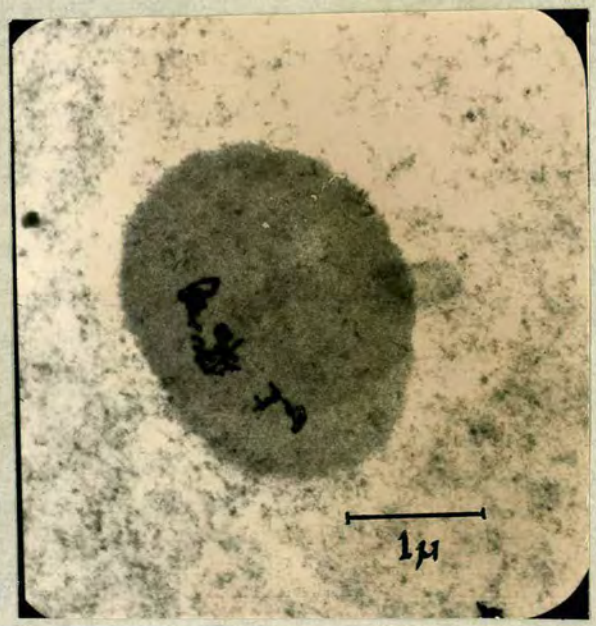


PLATE (31)

O-NU. 60 minute pulse + 6 hour chase

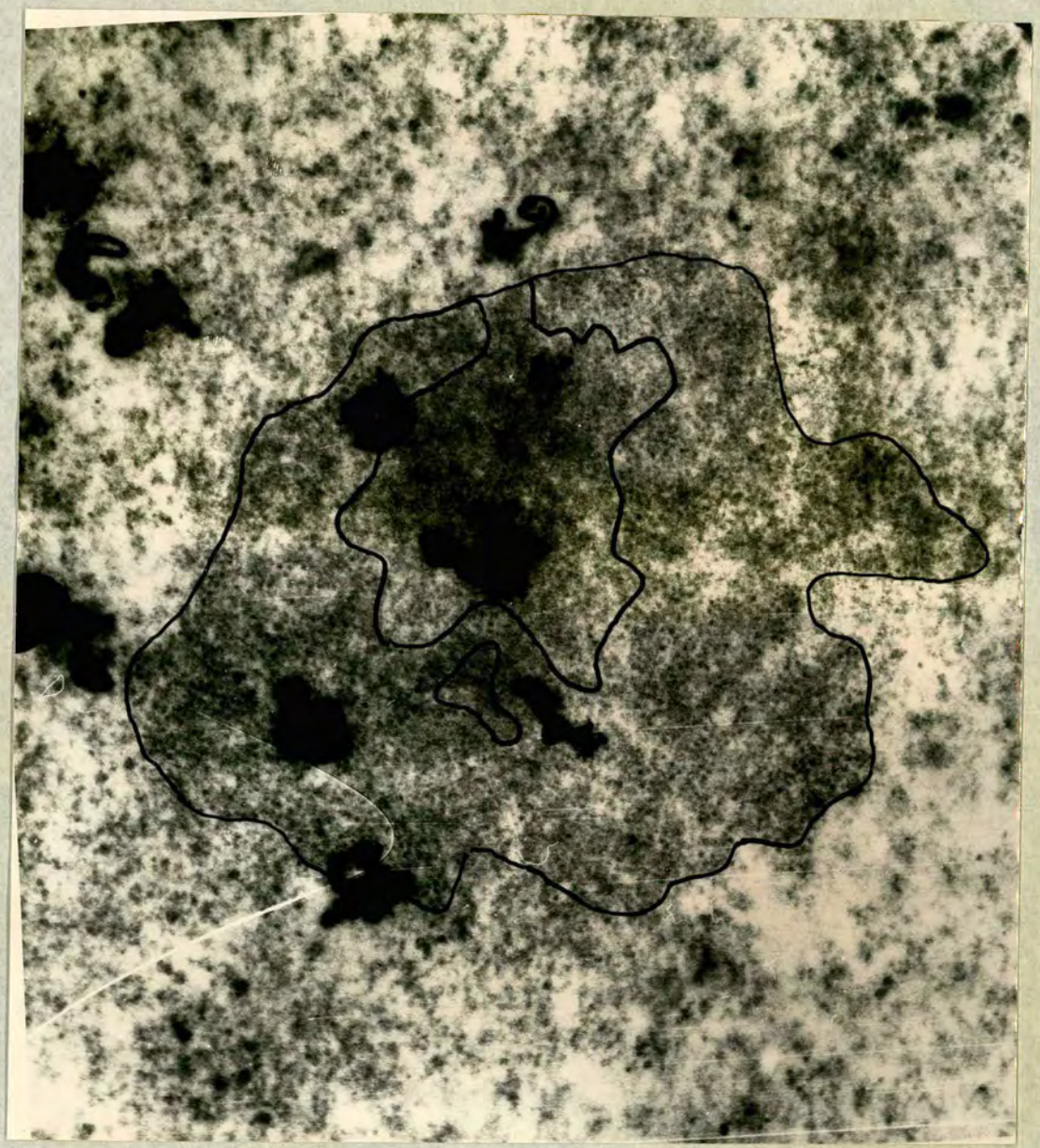


Plate (32). A sample tracing of a labelled nucleolus used in the estimation of specific activities, (see materials and methods, section 3.2).

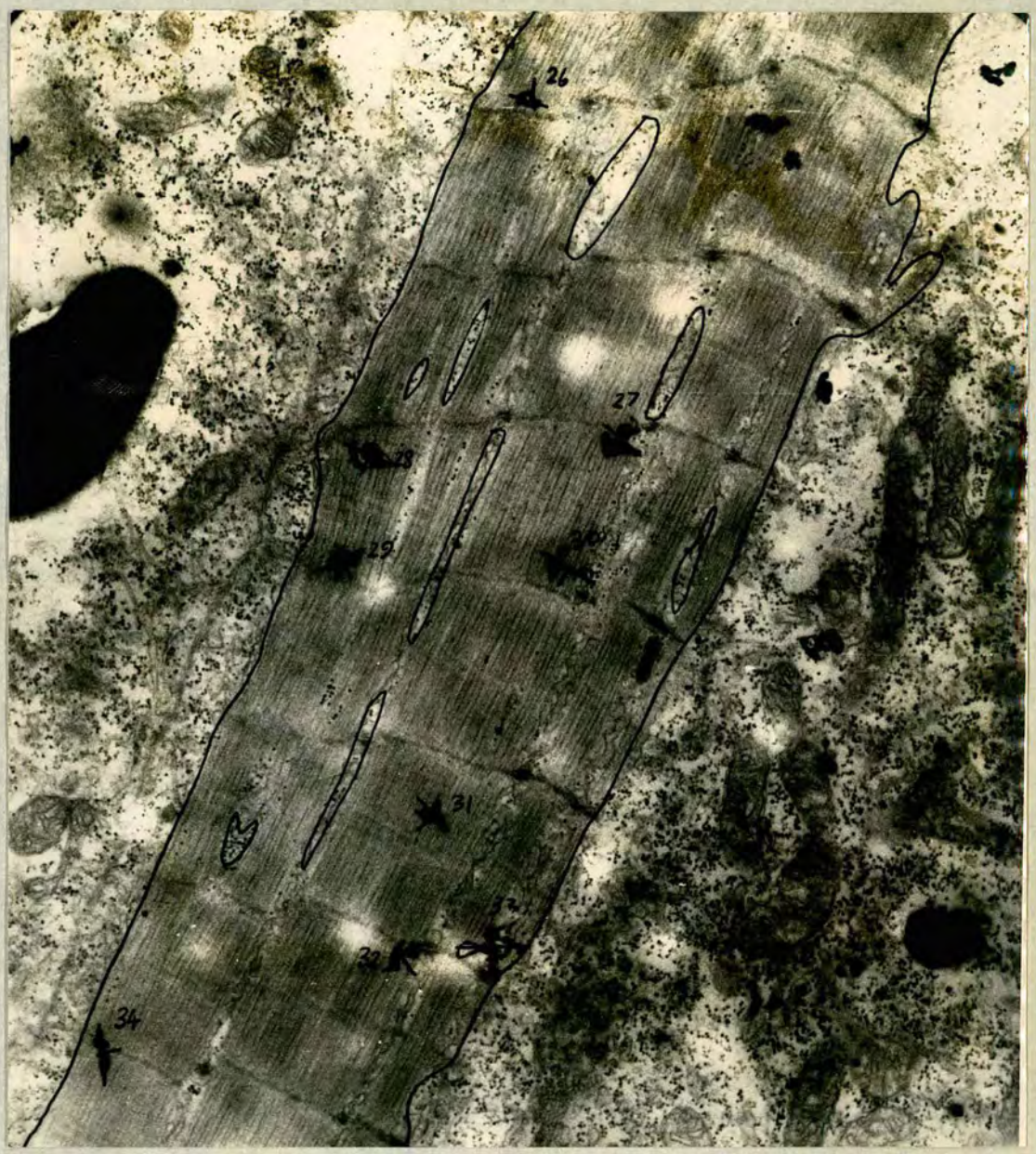


PLATE (33). A sample tracing of labelled myofibrils used in the estimation of specific activities (see materials and methods, section 3.2).

FIGURE 1. MUSCLE CELL COMPONENT SPECIFIC ACTIVITIES.TRITIATED URIDINE LABELLING. 12 weeks exposure
Blobs 18 weeks.

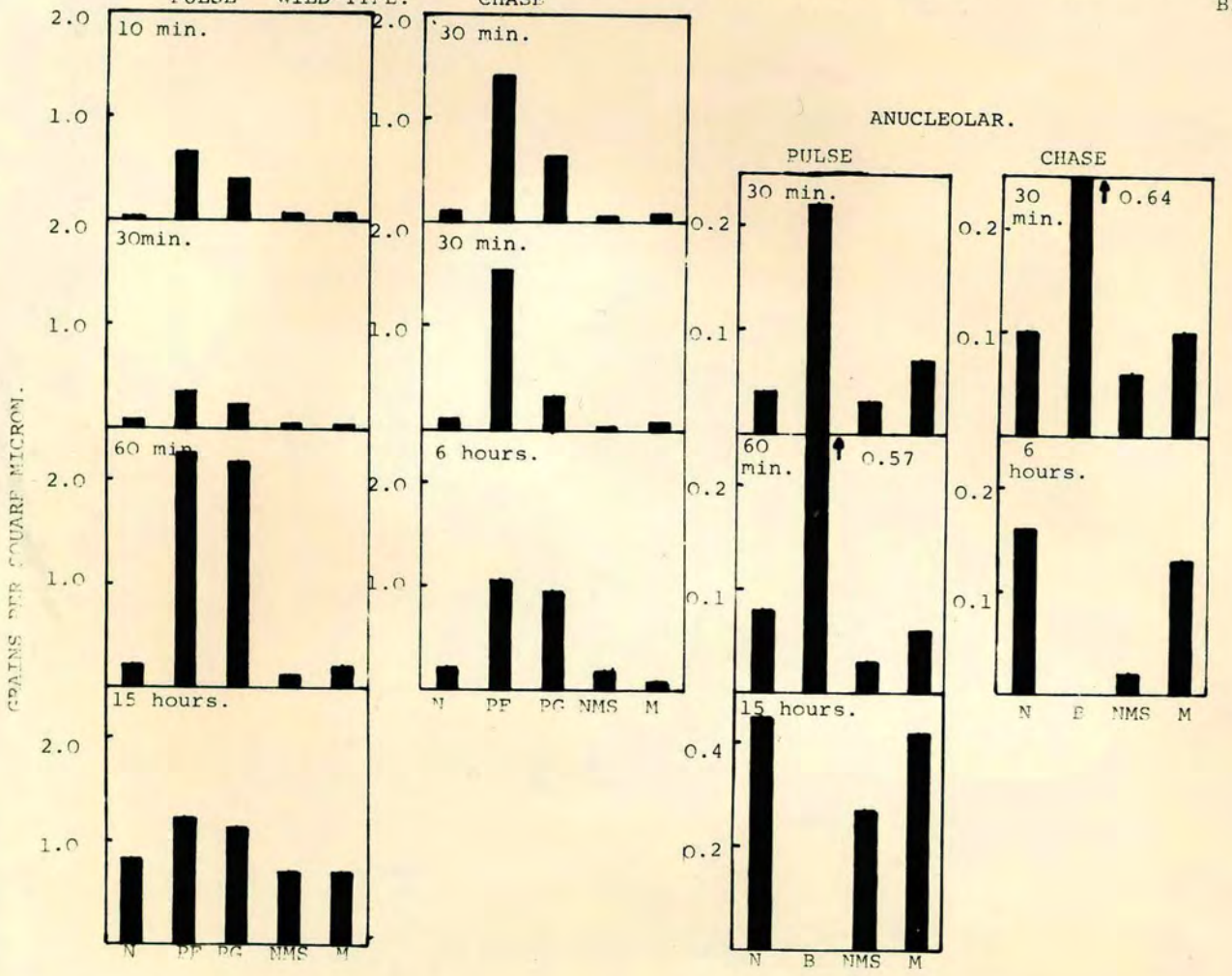
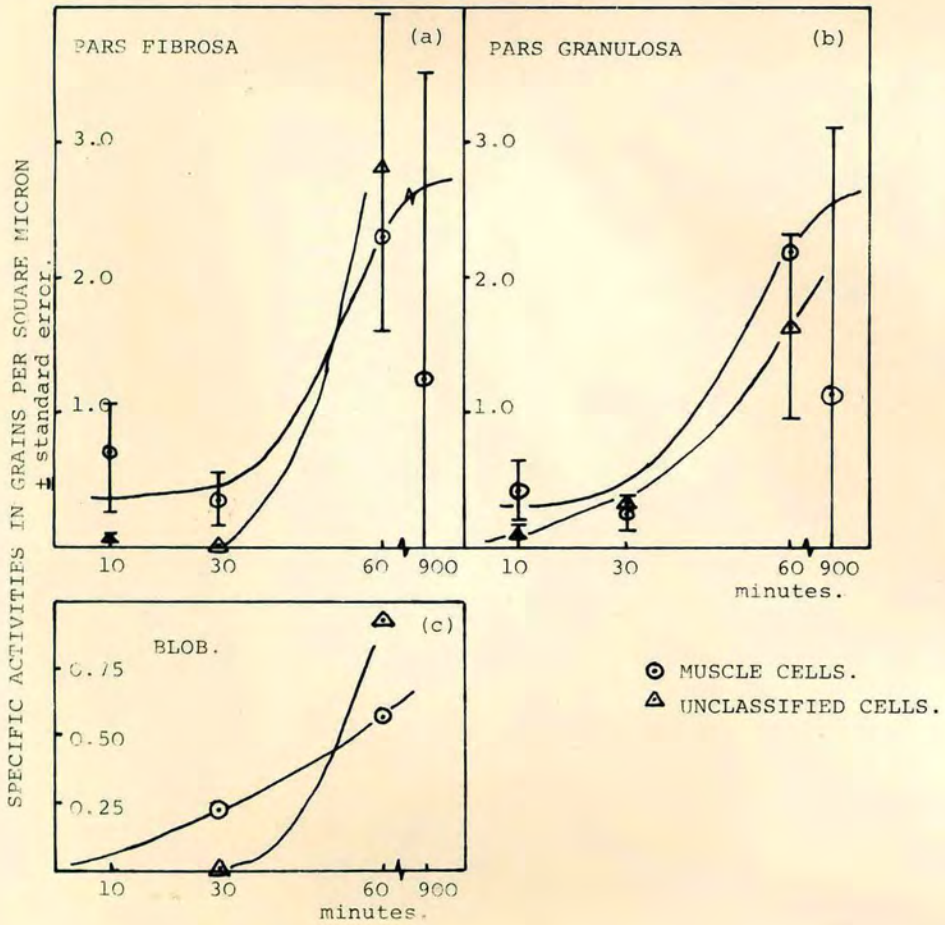


FIGURE 2. RATES OF UPTAKE OF TRITIATED URIDINE. 12 weeks exposure, (blobs 18 weeks).



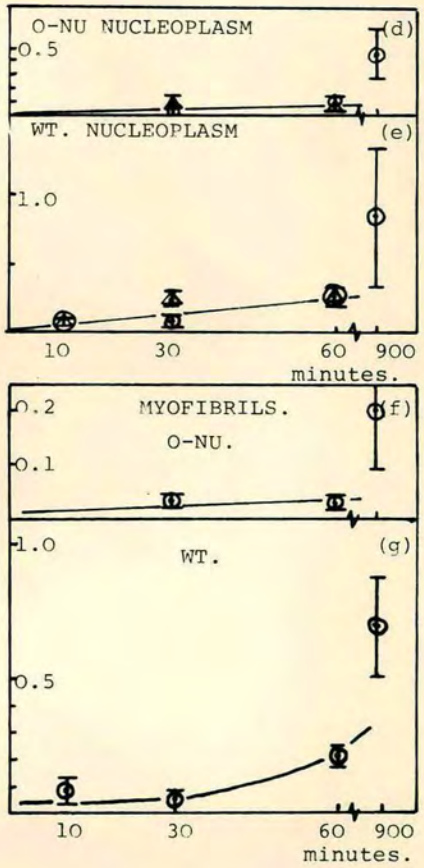


FIGURE 3. TRITIATED URIDINE LABELLING. SPECIFIC ACTIVITIES. 12 weeks exposure, (blobs = 18 weeks).

Ordinates = specific activity in grains/square micron ± standard error.

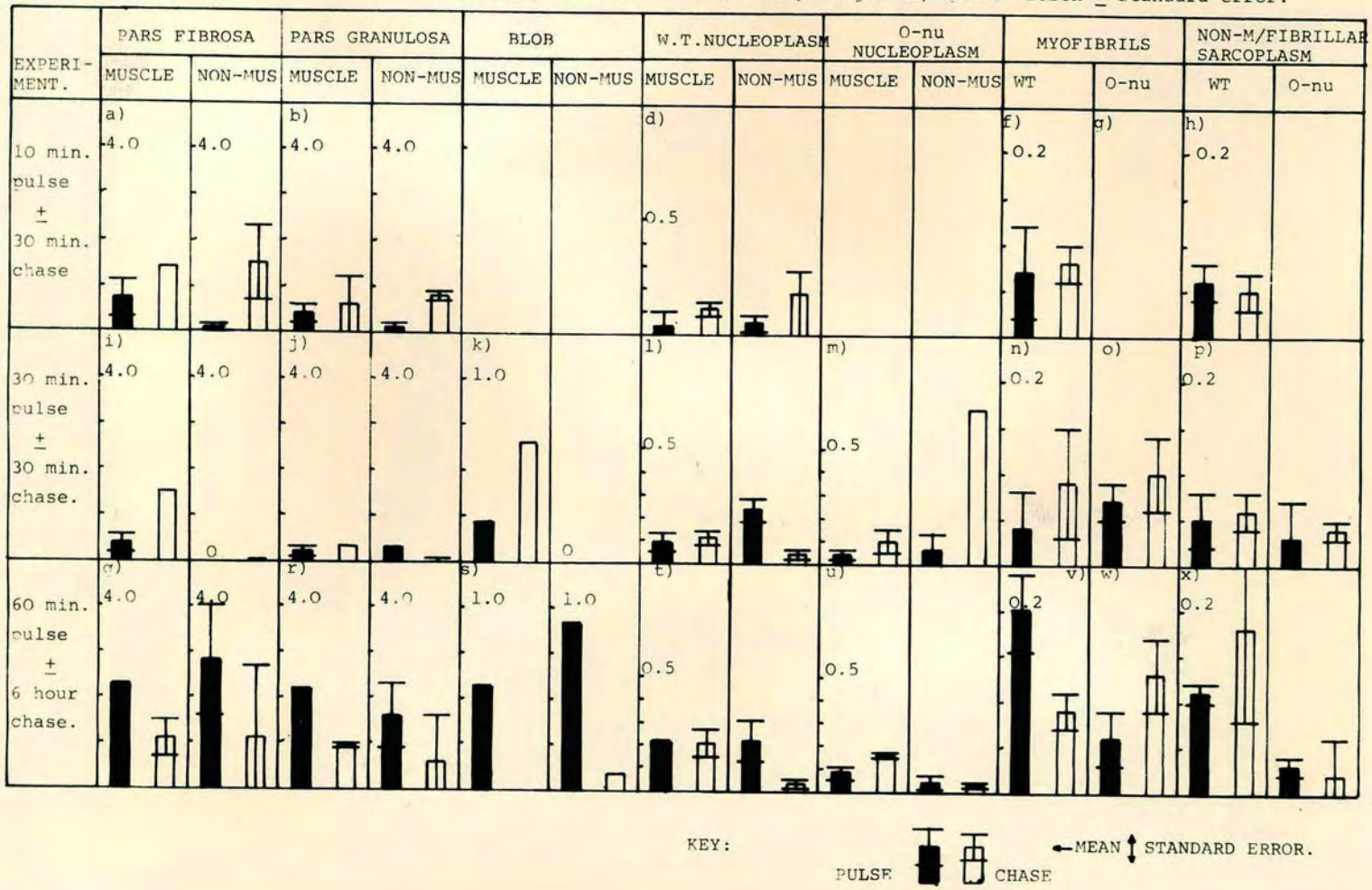


FIGURE 4. SARCOPMERE PROFILES.

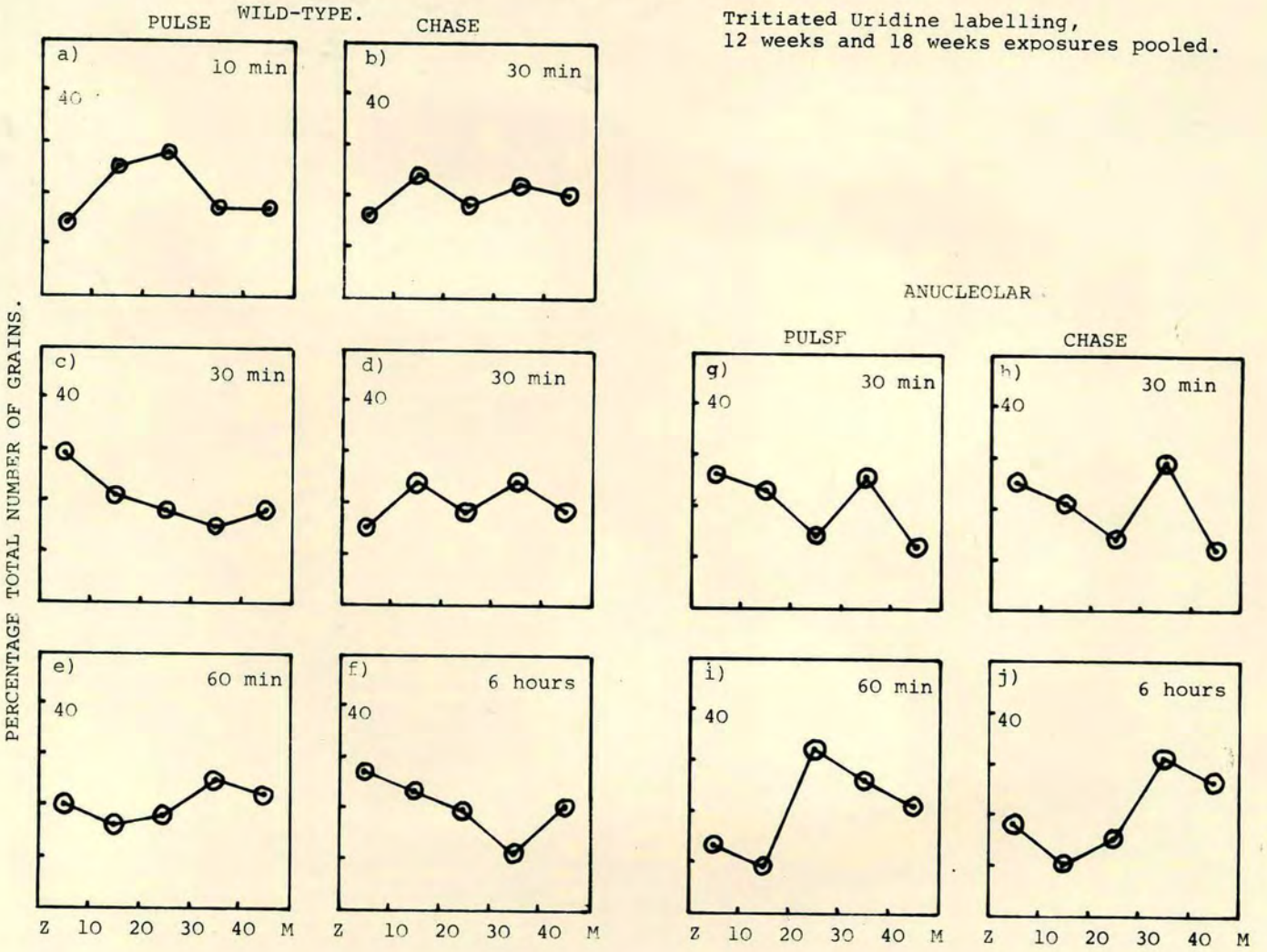


TABLE 1

12 weeks exposure autoradiogram specific activities and 95% confidence limits in grains/μm^2.

Expt	Cell	N	PF	PG	C	M
10+0	U+	0.05±0.03	0.04±0.07	0.08±0.09	0.01±0.02	-
	M+	0.03±0.07	0.66±0.22	0.40±0.22	0.06±0.02	0.07±0.05
10+30	U+	0.18±0.10	1.48±0.78	0.76±0.06	0.03	-
	M+	0.11±0.03	1.41	0.64±0.47	0.05±0.02	0.08±0.02
30+0	U+	0.23±0.05	0	0.32	0.07±0.09	-
	M+	0.09±0.04	0.35±0.20	0.24±0.12	0.05±0.03	0.04±0.04
	UO	0.06±0.07	-	-	0.03±0.04	-
	MO	0.04±0.02	-	-	0.03±0.01	0.07±0.02
30+30	U+	0.02±0.01	0.004±.001	0.01±0.05	-	-
	M+	0.11±0.03	1.53	0.33	0.04±0.01	0.09±0.06
	UO	0.68	-	-	0.01±0.005	-
	MO	0.10±0.05	-	-	0.06±0.02	0.10±0.04
60+0	U+	0.22±0.09	2.77±1.18	1.62±0.67	0.06±0.05	-
	M+	0.22	2.27	2.18	0.11±0.01	0.20±0.04
	UO	0.04±0.03	-	-	0.01±0.02	-
	MO	0.08±0.03	-	-	0.03±0.01	0.06±0.03
60+6h	U+	0.03±0.02	1.12±1.63	0.64±1.02	0.04±0.02	-
	M+	0.21±0.06	1.06±0.39	0.95±0.08	0.18±0.10	0.09±0.02
	UO	0.03±0.01	-	-	0.01±0.005	-
	MO	0.16±0.01	-	-	0.02±0.04	0.13±0.04
15+0 *	M+	0.84±0.51	1.23±2.25	1.14±0.65	0.69±0.33	0.69±0.18
	MO	0.45±0.18	-	-	0.27±0.03	0.42±0.24

*corrected from 4 week data

BLOBS 18 weeks exposure

Expt.	30+0*		30+30	60+0		60+6h
Cell	U	M	M	U	M	U
Specific Activity	0	0.22	0.64	0.92	0.57	0.08
% labelled	0	7	33	80	35	15

* corrected from 12 weeks exposure

3.4. DISCUSSION

A: Controls

The effects of RNAse, AMD and TCA were studied by light microscope autoradiography with thick sections of 12 to 15 hour pulsed material (wild type).

RNAse:- When pulse labelled material was lightly fixed in glutaraldehyde, well washed in phosphate buffer and then treated with RNAse at concentrations of 10 to 100 micrograms per ml for 1 to 6 hours at 37°, there was no appreciable reduction in the number of silver grains over the nucleus or cytoplasm compared with labelled material fixed in the normal way. Treatment with RNAse at 100 micrograms per ml. for one hour at 37° without prefixing abolishes silver grains although this may be a non-specific effect as the cells look necrotic.

Actinomycin D (AMD):- Preincubation with 1 microgram per ml. of AMD at 28° before labelling does not appreciably reduce the number of silver grains compared with untreated controls. When the same concentration is present during the whole of a 15½ hour pulse there are no silver grains (material pulsed without AMD is heavily labelled) although the effect may be non-specific as the tissue looks necrotic.

Trichloroacetic acid (TCA):- Pulse labelled material was prefixed in glutaraldehyde for 30 minutes and washed in phosphate buffer. Half of the material was treated with 5% TCA for 3x10 minutes at 0^{o} whilst the rest was kept in phosphate buffer. There was no appreciable difference in silver grain density between the two. TCA then does not remove the label.

The result with TCA shows that 3HU is being incorporated into a high molecular weight compound rather than sticking to cellular components in a non-polymerized fashion. This interpretation is supported by the sarcomere profile distributions of silver grains (see on) which show that the silver grains are distributed in a statistically significant non-random manner over the sarcomere.

The fact that RNAsed material still has silver grains over nuclei is not evidence that the 3HU is not in the form of RNA; massive amounts of RNA are synthesized in the nucleus and uridine, being on a direct pathway to RNA, must be incorporated into RNA more rapidly than any other compound. Therefore a very substantial amount of the nuclear label must represent RNA. The fact that RNAse does not noticeably reduce this labelling is evidence that the RNAse is not breaking down RNA, rather than evidence for the silver grains

not representing RNA. Perhaps these tissues contain an RNAse inhibitor not destroyed by glutaraldehyde, or perhaps the tissues are not permeable to RNAse. Wallace (1962) found that RNAse did not remove the pyronin affinity of O-nu myofibrils, which is consistent with this view, although the effect could be a non-RNA-specific effect of the stain. Also Jones (1971) successfully RNAsed 3HU labelled Xenopus somites using the same technique.

The AMD results probably show that the critical concentration and time of AMD treatment required to specifically inhibit RNA synthesis has not been found in these experiments. In one case the AMD treatment was insufficient to stop RNA synthesis and in the other case the treatment was so drastic as to cause cellular breakdown. Perhaps the concentration of AMD in nuclei and cytoplasm is kept ineffectively low by preferential absorption by other cell components. Jones (unpublished) found preferential uptake of 3H-AMD by yolk platelets in Xenopus embryos. Thus critical evidence for equating the presence of silver grains with the presence of newly synthesized RNA is lacking. Such evidence could perhaps be obtained by the extraction and characterization of incorporated 3HU or by RNAse digestion of glycol methacrylate embedded sections. However it seems

very likely that the silver grains on the autoradiograms do represent newly synthesized RNA.

B: General

(i) High Standard Errors.

There is frequently a high standard error about the mean grain density (e.g. Fig.3). There are four possible explanations for this. The first is that there may be a high non-uniform background grain density. This does not seem to be as the background is very low at 9×10^{-5} grains per square micron (appendix 7.3). The second possibility is that there is a high variation from one area of a grid to another in the sensitivity of the autoradiographic technique. This can be brought about by variations in thickness of the section, middle carbon layer and photographic emulsion. This type of variation will affect all regions of the cell equally and so the upper limit of this variation will be of the same order or less than the minimum variation observed throughout the experiment. This is probably as low as 6-7% of the mean as seen for example in 0-nu nucleoplasm, 60+0 (Table 1). The third possible source of variation is sampling error due to scoring small areas of low mean grain density. This is probably the major source of variation. This and the previous two types of variation can be kept to

a minimum by sampling a large area, or conversely a large number of grains. As large an area as conveniently possible was sampled. The actual areas sampled are recorded in appendix 7.2. The fourth type of mechanism giving high standard errors is real differences in specific activity between apparently similar structures under the same experimental conditions. This can be brought about by uneven penetration of the tracer or by real differences in the synthesis rates of apparently similar structures, caused for instance by asynchrony of the synthetic patterns of two cells. This fourth type of variation is also indistinguishable from that brought about by variation in ARG sensitivity, and the variation attributed to this has been shown to be about 7% of the mean.

The main source of variation then is in sampling error. Strictly speaking the only differences in grain density which can be usefully interpreted are those in which the standard errors do not overlap. Lesser differences are sometimes cautiously interpreted where several lines of evidence converge on the same conclusion. In such cases of comparison between say two similar structures under different experimental conditions many of the sources of error weigh equally in both cases.

(ii) Inefficiency of Chasing

There are many cases in the results of the specific activity of a structure increasing when chased with unlabelled medium after a pulse with labelled medium. Some of these cases are statistically significant, e.g. Figs 1a & b. The cautious interpretation is that RNA synthesized elsewhere during the pulse passes into the structure concerned during the chase. The counter-interpretation is that no such migration of RNA takes place but that the structure is utilizing a pool of 3HU or products of degrading RNA during the chase. There are two cases (WT non-muscle nucleoplasm in Figs 3 1 & t)of chasing significantly and markedly reducing specific activity. Thus as far as WT non-muscle nuclei are concerned, at least, there is no pool problem. It would be surprising if the nuclei of other tissues, having similar rates of synthesis (Figs 2 d & e), or even the cytoplasm, being in more intimate contact with the surrounding medium, were to have pool problems either.

It is assumed that within the time courses of these experiments there are probably no serious problems of interpretation due to a high specific activity of free 3HU remaining in washed cells during a chase, although again, conclusive evidence for this

is lacking. Such evidence could perhaps be obtained by measuring actual free pool activities during pulse and chase conditions by doing exhaustive TCA extractions on homogenates, provided enough material could be prepared.

C: Nucleoli

Nucleoli have the highest specific activity of the whole cell under all conditions (Figs 1 a-h), as is expected if, as in most cell types the bulk of RNA synthesized is rRNA (see introduction, 3.1). A six hour chase after a one hour pulse appears to decrease the sp.ac. of both components (Figs q&r). These reductions are not necessarily statistically significant but show the same tendency in the four separate cases in the figures. This tends to confirm rather than to deny the view that a large part of newly synthesized nucleolar RNA is lost and presumably exported to other parts of the cell. However, after short pulses chasing sometimes increases the sp.ac. of both components in muscle and non-muscle tissue (Figs 3a, b & i). It can be seen in the figures that this increase is statistically significant for non-muscle PF and PG (Figs 1a & b). This could be caused by some of the nucleolar RNA being synthesized in the nucleoplasm, as would be expected if the nucleolus processes mRNA or tRNA precursors

(see introduction 3.1). However there is no significant change in the sp. ac. of the nucleoplasm chased after a 10 min. pulse (Fig 3d). As the nucleoplasm occupies a much larger volume than the nucleolus it is possible for an undetectably small change in the sp.ac. of the nucleoplasm to produce a detectably large rise in nucleolar sp.ac. The curves in Figs 2a & b show a relatively low nucleolar sp.ac. up to 30 min. and then a marked significant rise at 60 min. to the steady state level as measured at 15 hours. This sigmoid type of curve is particularly marked in the PF of non-muscle cells (Fig 2a). The lag of the curve could well represent the synthesis time of a nucleoplasmic RNA which travels to the nucleolus. The activities present at up to 30 min. in the PF and PG will represent nucleolar RNA actually synthesized in the nucleolus. These conclusions are subject to the chasing being efficient. This problem is discussed in the general discussion (section 3.1 B), and although it seems unlikely that chasing is inefficient, this possibility cannot be ruled out.

The above conclusions concern the nucleolus as a whole. This seems the best way to interpret the data as the two components behave in a fairly parallel manner. The figures 3a, b, i, j, q & r show for

instance that there is never a clear cut case of a decrease in sp.ac. of one component during a chase coupled with an increase in the other as would be the case with a strictly precursor-product relationship. Instead, whenever one component increases its sp.ac. during a chase, so does the other, and the same happens with a decrease in sp.ac. The evidence then is for both components metabolising RNA independently. This would be the case if only the PG was synthesizing rRNA, rather than rRNA precursors being synthesized in the PF and transported to the PG. Other evidence for this has been discussed in the introduction (3.1A).

PF labelling could well represent a non-ribosomal function of the nucleolus. The labelling data shows an RNA product synthesized locally at a relatively slow rate and RNA synthesized elsewhere which temporarily accumulates in the PF. The work of other people, described in the introduction (3.1) indicates that this non-ribosomal RNA could be tRNA or even HnRNA or mRNA. This data is unable to distinguish between these types.

In the 60+60 hour experiments (Figs 3q & r) there are no significant falls in sp.ac. but in all cases there is an approximately 50% drop in mean sp.ac. Because of the consistency of this finding and despite

the lack of statistical significance it can be said that about 50% of newly synthesized RNA associating with nucleoli is stable in both components for at least 6 hours. This is so in both muscle and non-muscle cells. The results do not indicate whether this stable RNA is native or imported into the nucleoli.

This residual or slowly migrating nucleolar RNA could represent 28S rRNA which is known to remain in the nucleolus longer after synthesis than 18S RNA (see Birnstiel 1967). Again in this conclusion the possibility of inefficient chasing must be borne in mind.

D: Nucleoplasm

Again in short pulses there is a tendency for the nucleoplasm to increase in sp.ac. with chasing (Fig 3d) although this is not statistically significant and less marked than for nucleoli. This apparent increase then is probably not real and as discussed under 'Nucleoli' (last section) there may well be an undetectable drop in nucleoplasmic sp.ac. to account for the rise in nucleolar sp.ac. when chased after a short pulse.

WT nucleoplasm generally has a higher sp.ac. than O-nu nucleoplasm (Figs 2d, e and 3 l, m, t & u). The simplest explanation of this is that it is due to

the presence of ribosomal RNA in WT nucleoplasm. After 60 min. the sp.ac. of nucleoplasm has not reached the level of 15 hour pulsed muscle nuclei (there is no equivalent data on non-muscle cells) as shown in (Figs 2d & e). This, combined with the general lack of reduction of sp.ac. with chasing in muscle cell nucleoplasm (Figs 3d, l, m, t, u) indicates that over at least a 6 hour period there is a slow synthesis of RNA most of which is stable and stays in the nucleus, giving a steady build up of concentration to steady state values. In other words very little newly synthesized nucleoplasmic RNA travels to the sarcoplasm over a period of 6 hours. This is certainly the case in O-nu and hence is true for non-ribosomal RNA, but the data must be interpreted more carefully in WT. Here the apparent stability in nucleoplasmic RNA may represent a constant concentration of RNA in a state of flux, being supplied by the nucleolus which is falling in sp.ac. during this chase and hence loosing RNA, presumably to the nucleoplasm. As the sp.ac. of the nucleoplasm does not change, this nucleolar, presumably ribosomal RNA, might be rapidly travelling through the nucleoplasm and exiting to the cytoplasm. WT non-muscle nucleoplasm, however, shows a marked, significant decrease in sp.ac. when chased after 30 and 60 minutes (Figs 3 l & t), showing that the nucleolus cannot supply the nucleoplasm

with sufficient RNA to maintain its sp.ac. in a 30 minute chase. The conclusion from this is that muscle cell differentiation does involve some stabilization of ribosomal RNA is the nucleus. It is possible that this could be explained by muscle nuclei not dividing, or dividing more slowly than non-muscle nuclei. Otherwise the significance of this is not known. Another explanation could be that if some kind of informational RNA specific for myofibrillar protein synthesis is synthesized along with much other non-ribosomal RNA (e.g. HnRNA) in the nucleus then one way in which it could be protected from degradation and transported to the sarcoplasm would be to form a complex with a ribosome-precursor particle in the nucleus. Evidence for such possible associations in other cells is presented in the introduction (3.1A ii). The formation of such a complex may delay the exit of newly synthesized rRNA from the nucleus, conferring on it a relative stability. If this hypothesis is true then the ability of 0-nu embryos to develop normally in the absence of rRNA for protection purposes could be explained by postulating the re-entry of existing ribosomes into the nucleus for this purpose. This latter explanation is not favoured in view of the observations on myofibrillar labelling.

In this same time period of a 6 hour chase after a 1 hour pulse there is a marked rise in the sp.ac. of O-nu myofibrils whilst the NMS shows no significant change (Figs 3w & x). Thus both components of the cytoplasm show a rise or constancy whilst the nucleoplasm also shows a significant rise (Fig 3u). If the nucleoplasm was the sole precursor of sarcoplasmic RNA then a rise in the sp.ac. of the sarcoplasm during a chase would be matched by a very large fall in the sp.ac. of the nucleoplasm because it has a much smaller volume than the sarcoplasm. Fig. 3t shows that WT nuclei do lose most of their activity after a 6 hour chase, due to processing and exit of rRNA, showing that chasing is not necessarily inefficient. Thus it seems very unlikely that nuclear non-ribosomal RNA, synthesized in periods up to one hour, is the precursor of non-ribosomal RNA associating with myofibrils.

This is in very good agreement with the observations of other workers that HnRNA does not precurse mRNA (Introduction 3.1). As with their findings these results do not preclude the possibility that a small fraction of HnRNA is a precursor of some cytoplasmic mRNA. However, in these experiments nuclear labelling does not account for all of the label appearing in muscle cytoplasm.

There is some evidence (Chapter 4.1) that DNA is the cytoplasmic informational molecule originating in the nucleus in chick muscle cells.

In the 60+6 hour experiment in WT the large drop in nucleolar sp.ac. is not matched by a rise in nucleoplasmic sp.ac. It is probable then that ribosomal RNA slowly passes through the nucleoplasm over a 6 hour period and is taken up by the relatively large volume of the NMS, which shows a small, insignificant rise in sp.ac. (Figs 3q, r, t, & x).

E: Blobs

The data for blob labelling was not submitted to statistical testing because of the very high frequency of unlabelled blobs in all the experiments (Table 1) rendering the statistical method invalid. It is obvious though that the standard error is high. Blobs label at rates comparable with other structures (Fig.2) except nucleoli which label much faster than all other structures. Where comparisons can be made it can be seen that blobs label similarly to the PF but at much lower absolute level, (about 25% as shown in Figs 2a & c), in that there is a 30 minute lag in blob labelling and in PF labelling in non-muscle cells, but in muscle cells these structures appear to label from zero time. In the PG labelling

appears to start at zero time in all cell types. In non-muscle cells then it appears that blobs and to a greater extent PF obtain RNA synthesized elsewhere, about 30 minutes after synthesis, whereas muscle cell blobs and PF make at least some of their own RNA. Probably muscle cell blobs also take in RNA made elsewhere because blobs increase in sp.ac. when chased after a 30 minute pulse (Figs 3i & k). This is not necessarily statistically significant but is supported by the same finding in muscle PF, (Fig 3i). Over a period of 6 hours at least non-muscle blobs, like both nucleolar components, lose most of their RNA (Fig.3s). The ultrastructural and developmental similarities of blobs and PF are discussed in the introduction (3.1A iii). The 3HU labelling evidence supports this similarity. It cannot be said from the data what type of RNA the labelling represents. The component of the RNA behaving similarly in all cell types could represent the synthesis or transport (e.g. for the purpose of methylation) of tRNA or of mRNA species common to all cell types. The component unique to muscle cells, that is apparently synthesized in situ or transported to the PF or blob within 10 minutes of synthesis, may represent mRNA precursor for myofibrillar proteins. The high percentage of blobs in all experiments having no silver grains

(Table 1) could have three causes. One is discontinuous function in time. That is the aquisition of RNA, either autosynthesized or accumulated from elsewhere takes place in discontinuous short bursts throughout the time of the experiment so that at a given time only a few blobs are labelled. This seems likely as the percentage of labelled blobs would be expected with this explanation to increase with overall grain density and this is the case (Table 1). The second possible cause is high sampling error because of the smallness of the area of the blobs (see e.g. Plates 14 & 27 and appendix 7.3). In other words, even with a high concentration of 3HU (in the form of RNA) in each blob, the probability of a tritium disintegration leading to a silver grain during the exposure time may be less than unity in the small area involved. Again this explanation has the expectation of an increased percentage of labelled blobs with increased grain density as is the case. The third possible cause is that not all the blobs, as sectioned, are involved in RNA metabolism. This theory is strengthened by the observation that where grains occur on blobs they are frequently situated on the extreme edge (see for example the pronounced asymmetry of labelling in some blobs as in Plates 19 and 31). Asymmetrical labelling of blobs was also observed frequently by Jones (1971). Hay & Gurdon

(1967) and Jones (1965) occasionally observed asymmetric granular caps on blobs. Thus if only part of the blob is active in RNA metabolism then not all of the sections would necessarily contain this area, particularly as it seems that this area is only a portion of the surface of the blob. The data is not able to distinguish between these alternative explanations.

F: Myofibrils

At 60 minutes of pulse time WT myofibrils are labelling at a much faster rate than 0-nu myofibrils (Figs 2f & g). Assuming that myofibrils grow at more or less the same rate in the two genotypes it seems likely that myofibrils take up ribosomes (newly synthesized, in part, in the case of the WT) The uptake of 0-nu myofibrils is linear up to 60 minutes whereas the WT myofibrils increase the rate of uptake between 30 and 60 minutes. This may indicate that ribosomes only attach to myofibrils after 30 minutes after their synthesis begins. This is to be expected for ribosomes synthesized elsewhere; in the nucleolus.

WT myofibrils have a sp.ac. 700x background after a mere 10 minute pulse (Fig 3f). This is stable for at least 30 minutes. Even taking a higher background as a base, that of 10 minute pulsed non-muscle

cytoplasm (Table 1, line 1), thus allowing for such possibilities as inefficient washing out of unincorporated 3HU, mitochondrial RNA synthesis (always counted as part of the cytoplasm or NMS) and binding of 3HU to general protein (although this seems unlikely from the TCA control), myofibrils still have a sp.ac.100x background. Could non-polymerized 3HU be binding to a particular myofibrillar protein? This seems unlikely for three reasons. The first is again the TCA control. The silver grains represent tritium covalently attached to a high molecular weight compound. Secondly 10 minute pulsed NMS has a similar sp.ac. to myofibrils (Fig. 3h). NMS does not presumably have similar concentrations of myofibrillar proteins to myofibrils themselves, and so binding of 3HU to myofibrillar protein does not explain the high sp.ac. of NMS after 10 minute pulse. Thirdly, the graphs of grain distribution along sarcomeres in Figs 4 & 5 have a variable shape. For instance the X^2 values calculated in appendix 7.2 show that curve 4b is not significantly different from a horizontal straight line, whereas curve 1h is significantly different from a horizontal straight line. i.e. the grain distribution along sarcomeres significantly changes from one experiment to another. As the distribution of the major myofibrillar proteins at least presumably does not change it seems unlikely that

the 3HU is binding to a particular myofibrillar protein. There remains the possibility that muscle cells, unlike non-muscle cells, are difficult to wash free of unincorporated 3HU, thus giving a high, uniform background over muscle cells. The sarcomere profiles of Fig.4 again make this explanation very unlikely, as this would lead to horizontal straight lines with low X^2 values in all of the pulse experiments.

There are other direct tests possible for the specific demonstration of mRNA in myofibrils. The first is the direct visualization of myosin synthesizing polysomes in myofibrils by EM. This is discussed and demonstrated in Chapter 2. The second, more specific test would be the use of <u>in situ</u> hybridization of nucleic acids. This technique allows the detection of poly-A sequences (specific to mRNA and HnRNA molecules) in cytological preparations. Preliminary experiments on these lines are presented in Chapter 5. The use of reverse transcriptase to synthesize highly radioactive DNA complementary to purified myofibrillar protein mRNAs would also allow this technique to be used on muscle tissue. This technique has been used to detect the sites of 9S-globin mRNA in mouse embryonic liver erythroid cells (Jones, in prep.).

Thus, although the evidence is not conclusive, the rapid labelling of myofibrils suggests a case for cytoplasmic transcription occurring, i.e. RNA is being transcribed from a DNA template situated in either the myofibrils themselves or in the cytoplasm surrounding them. As was shown in the discussion of nucleoplasmic RNA metabolism, little or no detectable non-ribosomal RNA travels out of the nucleus in periods of up to 6 hours. If the nucleus was the site of a non-ribosomal RNA precursor to that associating with myofibrils, the excess volume of the myofibrils to nuclei would produce a tremendous reduction in nuclear sp.ac. in a chase, and this is not so. The data again suggests then that the non-ribosomal RNA associated with myofibrils is not nuclear in origin and that transcription is taking place in the myofibrils or surrounding sarcoplasm. This interpretation is subject to the reservations concerning labelling specificity discussed in the 'controls' section above, and is fully discussed together with independent evidence in the final discussion (Chapter 7.2).

That this rapidly labelling myofibrillar RNA is non-ribosomal is demonstrated by the fact that it appears on WT myofibrils before nucleoli lose label in a chase and that 0-nu myofibrils also become stably radioactive after 30 minutes (Fig.3o).

Comparing WT and O-nu 1 hour pulses ± 6 hour chases, it is seen that O-nu myofibrils incorporate about half the radioactivity of WT in 1 hour (Figs 3v & w) but that all of this is stable for at least 6 hours, whereas the excess sp.ac. of WT myofibrils is unstable (temporally or spatially). Probably this excess sp.ac. of WT myofibrils is due to newly synthesized ribosomes and that these do not stably attach to myofibrils but can be chased off over a period of 6 hours. This is to be expected from the widely supported view that ribosomes travel along the mRNA and detach from one end (e.g. Risebrough et al 1962).

So two populations of myofibril-associated RNA have been resolved by their distinctive kinetics.

Ribosomes take about 30 minutes to be synthesized in the nucleolus and become attached to the myofibrils, and less than 6 hours to become detached from them. Non-ribosomal RNA, which may be synthesized in the myofibrils, or more likely in the surrounding cytoplasm, attaches to the myofibrils, and remains stably attached for at least 6 hours.

Can the locations of these different species of RNA be determined from the sarcomere profiles of Fig.4? The high X^2 values for some of these curves, shown in

appendix 7.2 indicate statistically significant deviations from a horizontal straight line, which suggests that they can be located.

The loss of ribosomes should be seen by comparing WT 60+0 (Fig 4e) with WT 60+6H (Fig 4f) as it has been shown that with these conditions ribosomal RNA chases off myofibrils leaving behind the stable non-ribosomal RNA. In the latter a relative drop is seen at the side of the M line, (position 35) where there was a small peak in Fig 4e, an observation supported by there being a rise at the Z end; (as the graphs represent percentage of total grains observed in an experiment, a real fall in the number of grains in one region of the sarcomere will produce the dual effect of a fall in the graph there and a rise in the rest of the line). Ribosomes appear to detach from either side of the M line.

The acquisition of ribosomes should be shown by comparing WT 30+30 with WT 30+0 (Figs 4c & d). There is a large rise in myofibrillar sp.ac. in WT at 60 minutes and it has been shown that this gain is due largely to ribosomes. By chasing for the second half of this period a more clear cut picture should emerge of ribosome attachment. The graphs show that this chase lowers the curve at the Z end and raises a peak

again on either side of the M line at position 35. Ribosomes then appear to both attach and to detach from the sarcomere at positions on either side of the M line, corresponding to either the ends of the actin filaments, (the positions of which vary in this method of data presentation because of the varying states of contraction of the myofibrils) or on either side of the middle of the myosin filaments. This agrees with Larson's theory (Larson et al 1969) that ribosomes are stationary on the sarcomere.

Non-ribosomal stable RNA should be shown in O-nu 30+30 in Fig. 4h. This graph again has a large peak on either side of the M line and an upward trend from a quarter of the distance along the sarcomere towards the Z line. The persistence of these trends in the longer 6 hour chase in Fig.4j shows that this stable RNA probably remains at fixed positions on the sarcomere; on either side of the M and Z lines. The site of synthesis of this RNA should be shown in O-nu 30+0 (Fig 4g) which is virtually the same as the chase(Fig. 4h). An even better indication of the synthesis site should be the shorter pulsed WT 10+0 (Fig 4a), which, although WT will not yet have acquired newly synthesized ribosomes. This shows a broad peak on either side of the Z line (position 15 to 25). This rapidly flattens off in a 30 minute chase (Fig 4b)

to give the beginnings of the peak to the sides of the M line at position 35. Probably, then non-ribosomal RNA attaches to or is synthesized mainly near the Z lines and partly migrates to the M line, passing rapidly over the intervening distance, giving the stable double peaks near or on the M and Z lines seen on all of the O-nu graphs in Fig.4.

3.5. SUMMARY

Evidence has been presented for the following:
(1) Nucleoli as well as synthesizing RNA, take in RNA from the nucleoplasm, particularly noticeable in the pars fibrosa. This could represent the processing of mRNA or t RNA precursors.
(2) Blobs have a similar labelling pattern to the pars fibrosa but at a much lower level. Non-muscle cell blobs take in RNA with a synthesis and/or processing time of about 30 minutes synthesized elsewhere, presumably in the nucleoplasm. Muscle cell blobs also do this and in addition make their own RNA. Most of one or both types of blob RNA chase out in under 6 hours. The destination of this RNA, or if indeed it gets anywhere before breakdown, is not known, but this supports the view that part of the nucleolar function, morphologically represented by the blob, is the processing of nucleoplasmic RNA.

(3) Muscle cell differentation involves the stabilization of nucleoplasmic ribosomal RNA or ribosomal RNA precursor. The significance of this is not known.

(4) Over a period of 6 hours the bulk of newly synthesized nucleoplasmic non-ribosomal RNA in muscle cells, stays in the nucleus. An insufficient amount of label chases out of the nuclei to account for cytoplasmic labelling.

(5) Non-ribosomal RNA is synthesized in the sarcoplasm and becomes associated with the myofibrils.

(6) This RNA is synthesized near Z lines, or in the non-myofibrillar sarcoplasm and attaches to the Z lines, and some of it travels towards the M line. It then stabilizes in these positions.

(7) Ribosomes start leaving the nucleolus 30-60 minutes after synthesis begins, travel through the nucleoplasm, and attach to myofibrils.

(8) Ribosomes attach to myofibrils on either side of the M line and stay in this position until they detach within 6 hours of attaching.

(9) These conclusions rest mainly on two assumptions. One is that the silver grains in the autoradiograms exclusively represent newly synthesized RNA and not tritiated uridine in some other form, and the other

is the efficiency of chasing after pulse labelling is high. Although the justifications presented for these assumptions are fairly strong, they are not conclusive. Final confirmation of these theories await the results of the types of experiments mentioned in the text.

CHAPTER FOUR

DNA METABOLISM IN EMBRYONIC XENOPUS MUSCLE TISSUE

4.1. INTRODUCTION

DNA metabolism in muscle cell nuclei has been partly discussed in Chapter 2.1. The conclusion is that once differentiation is well under way DNA synthesis and mitosis has stopped. It is debatable whether or not the cessation of DNA synthesis is a trigger for differentiation to commence. This chapter is concerned with cytoplasmic DNA metabolism. Winnick & Winnick (1960) reported a DNA content of 0.3% of protein weight in myofibrils isolated from embryonic chick muscle cells and assumed this to be due to

nuclear contamination. It may have been due to mitochondrial contamination. Cytoplasmic DNA is commonly found in animal cells in mitochondria (Rabinowitz & Swift 1970). In dystrophic mouse muscle (Appendix 7.1) an RNAse resistant nucleic acid was found to coextract with myosin. This substance has not been sufficiently characterized. Bell (1969) labelled embryonic chick muscle tissue with tritiated thymidine (3HT) and extracted from the cytoplasm a labelled 16S particle which contained a 7S nucleic acid. The 7S peak appeared in the cytoplasm after short pulses, as if it is a precursor of the 16S particle. This was claimed not to be due to nuclear contamination from the results of experiments in which nuclei were isolated after labelling and then added back to unlabelled cytoplasm: the 16S peak did not appear. Tissue labelled with tritiated uridine (3HU) also produced a 7S peak which was DNAse resistant and so is presumably RNA. When various cytoplasmic fractions from 3HT labelled tissue were deproteinized and analyzed, the 0-45S fraction yielded a 7S labelled nucleic acid, the 75-120S fraction yielded a labelled 14S nucleic acid and the heaviest fraction yielded a spread of radioactivity centering on 14S. Bell claimed the 7S species to be informational DNA (I-DNA), that it associates with protein to form a 16S particle, that possibly before

this occurs RNA is transcribed from I-DNA and that the I-DNA can polymerize in association with polysomes forming a DNA-RNA protein synthesizing complex. Unfortunately the DNAse sensitivity of the 7S species is not mentioned in this paper, so it is possible that the I-DNA is really RNA, the thymidine having been converted to uridine before incorporation. It is unlikely that he is observing mitochondrial DNA because of the slow sedimentation of the particle (16S) and the apparent association of the 7S species or some other thymidine labelling substance (possibly a polymer of 7S DNA) with polysomes.

In more recent work Bell (1971) showed that this cytoplasmic thymidine incorporation is sensitive to DNAse but not to RNAse and that I-DNA has a different buoyant density to nuclear DNA, mitochondrial DNA and RNA, Also the synthesis of nuclear DNA and I-DNA are differentially affected by various inhibitors of DNA synthesis such as hydroxyurea, fluorodeoxyuridine and cytosine arabinoside. At certain concentrations actinomycin D enhances I-DNA synthesis whilst not affecting nuclear DNA synthesis and ethidium bromide is claimed to inhibit mitochondrial DNA synthesis at lower concentrations than those required to inhibit I-DNA synthesis (Chepelinski & Bell 1972). These

results, although interesting, do not prove that the so called I-DNA is not a result of nuclear damage, as is claimed for the rapidly labelled DNA extracted from mouse liver (Williamson 1970). In these cells the yield of cytoplasmic DNA is almost abolished if the extraction is performed on fresh liver rather than liver cells cultured for several hours in labelled medium. Autoradiography of labelled cells shows that many of the cells with labelled cytoplasm have distintegrated nuclei. However, culturing these cells enriches the population of erythroid cells, which perhaps accounts for the diminution of the yield from fresh liver, and these cells normally extrude their nuclei at a later stage of development, so that the results are not directly comparable to those of Bell.

The results of Bell's group were received with scepticism by much of the scientific community, probably mainly because of the way in which the results were interpreted. There is virtually nothing in his work which justifies the use of the term 'informational DNA', as experiments testing the informational content of the DNA have not yet been reported. However, the results, at face value, are at least a cause for some concern in assuming that the 'central dogma' of molecular biology is true for all systems. In an

entirely different system, virus infected human cells, the 'central dogma' has in fact been inverted by the discovery of RNA-dependent DNA-polymerase (Gallo et al 1971). Jones (unpublished results in this laboratory) pulsed Xenopus embryo somitic muscle with 3HT for 12 hours and submitted the tissue to EMARG. There was extensive labelling of the cytoplasm, including the myofibrils, distinctly located from the mitochondrial labelling. No controls were performed (the unexpected result was itself meant to be a control in a uridine labelling experiment), so it was not known whether the label represented RNA or DNA. This work was extended in the experiments described here.

4.2. MATERIALS & METHODS

The somite regions of ten wild type 2nd form tadpoles, 6 days old (stage 22, Rugh 1948) were removed as in chapter 3.2, each cut transversely into three pieces and washed and stored in sterile 1x Minimal Eagles Medium (Hanks based plus non-essential amino-acids, Wellcome) + 0.1% BSA (Bovine Albumen Powder, Fraction V from Bovine Plasma, Armour Pharm. Co. Ltd.). This medium is referred to as MEM. The somite pieces were then placed in 4.75ml of MEM + 0.25ml of a solution of tritiated thymidine in water (methyl ^{3}H-thymidine, 19 Ci/mM, 1mCi/ml., Radiochemical Centre, Amersham),

making a final specific radioactivity of 50μCi/ml., in a sterile screw topped 20 ml. glass bottle and incubated at 15°C. At intervals of 10 minutes, 1, 6 and 12 hours a few pieces of tissue were removed and washed twice, briefly in fresh MEM and either fixed in glutaraldehyde as in Chapter 3.2 or chased for various times by incubating in fresh MEM at 15°C and then fixed. Another few pieces of tissue were incubated in identical radioactive medium but containing 1μg/ml of AMD (Dactinomycin, Lysovac Cosmogen, Merck, Sharpe & Dohme), for 12 hours and then washed and fixed in the same way as the others. All pieces of tissue were, after glutaraldehyde fixation, washed in 3 changes of phosphate buffer (Chapter 3.2) for at least 8 hours at 4°C to remove non-incorporated label and fixative. Two of the pieces of 12 hour pulsed tissue (minus AMD) were placed in separate tubes containing 1ml. of MES/Mg buffer (0.05M 2-(N-morpholino)-ethane sulphonic acid (Sigma); 0.002M magnesium acetate, adjusted to pH 7.0 with sodium hydroxide). To one tube 20μg of electrophoretically purified DNAse (DN-EP, Sigma) was added and the two pieces were incubated at 37° for 30 minutes. To the DNAse sample two further additions of 20μg of DNAse were added at 10 and 20 minutes of incubation. The two pieces were then washed in 5%TCA (trichloroacetic acid in water) for 5 minutes at 0°C. All of the pieces were washed in phosphate buffer for

at least another 12 hours at 4°C. The pieces were then fixed in osmic acid and embedded in Araldite as in Chapter 3.2. Thick and thin section autoradiograms were prepared from the material for light and electron microscopy as in Chapter 3.2. Thick section ARGs, after exposure and processing, were stained in 0.2% toluidine blue, dried, mounted in immersion oil and photographed on a Zeiss microscope using x100 oil immersion and x40 objectives.

4.3. RESULTS

PLATE (1). Electron microscope autoradiogram of wild-type Xenopus somites after a 6 hour pulse with tritiated thymidine followed by a 6 hour chase. 12 weeks exposure. Myofibrils and mitochondria are both labelled.

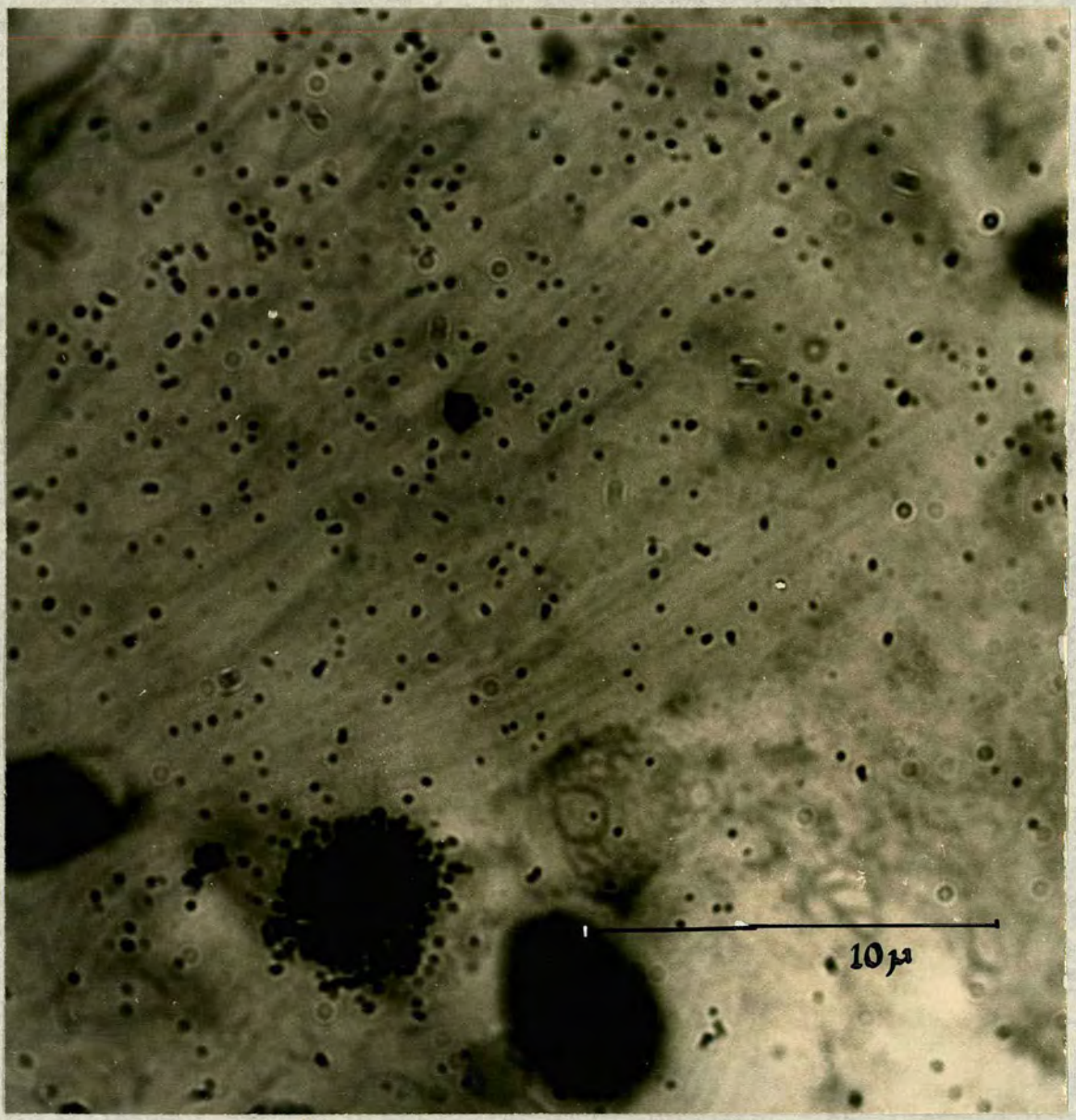


PLATE (2). 12 hour pulse of 3HT. 2 weeks exposure. Plates 2-6 are light microscope autoradiograms. Myofibrils, and probably nuclei are labelled.

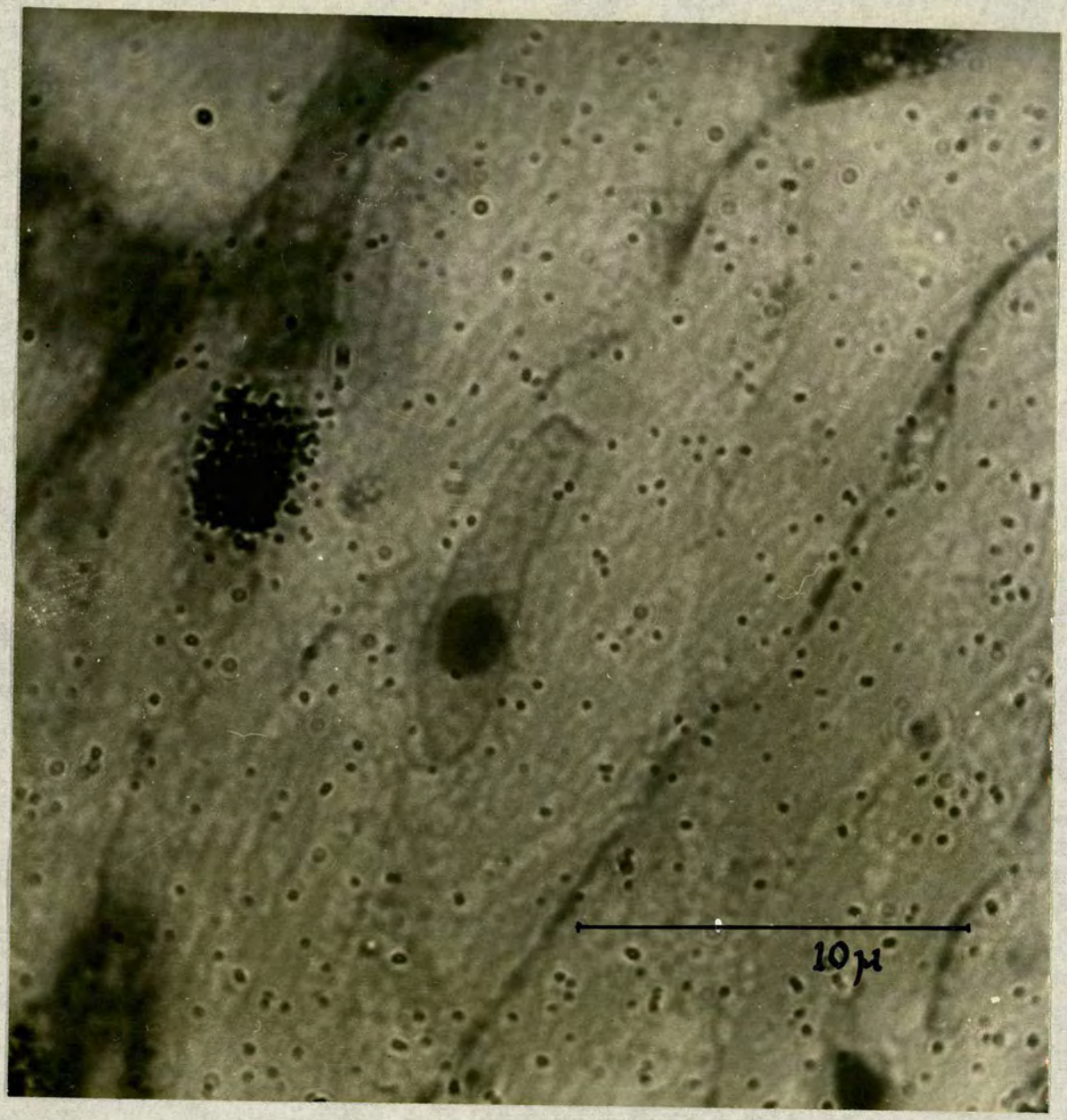


PLATE (3). 12 hour pulse of 3H-T, TCA washed. 2 weeks exposure. Myofibrils are labelled, and one of the two nuclei.

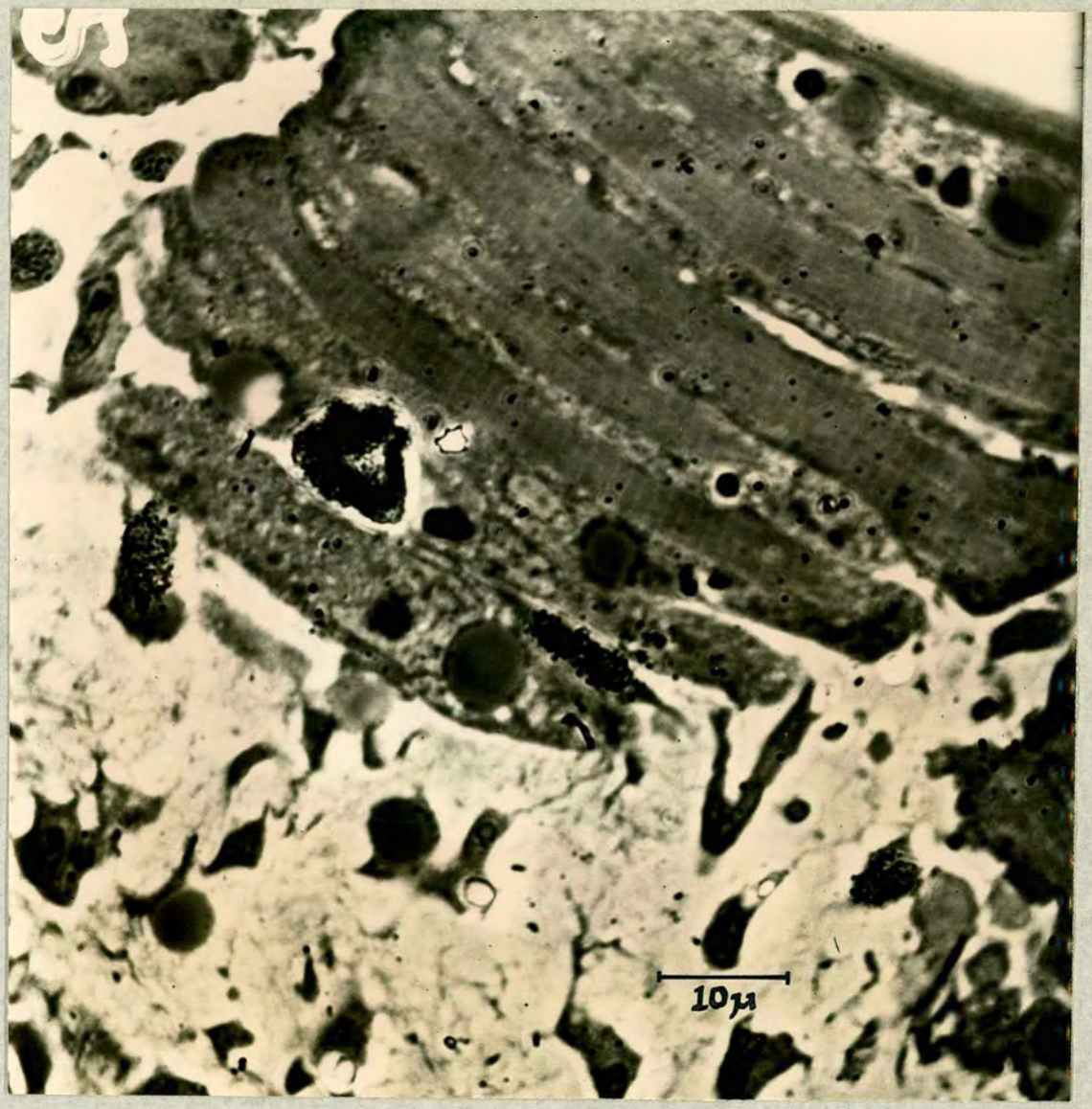


PLATE (4). 10 minute pulse of 3H-T. 4 weeks exposure. Some nuclei are labelled in muscle and non-muscle tissues. Myofibrils are lightly labelled. All other cytoplasm is unlabelled.

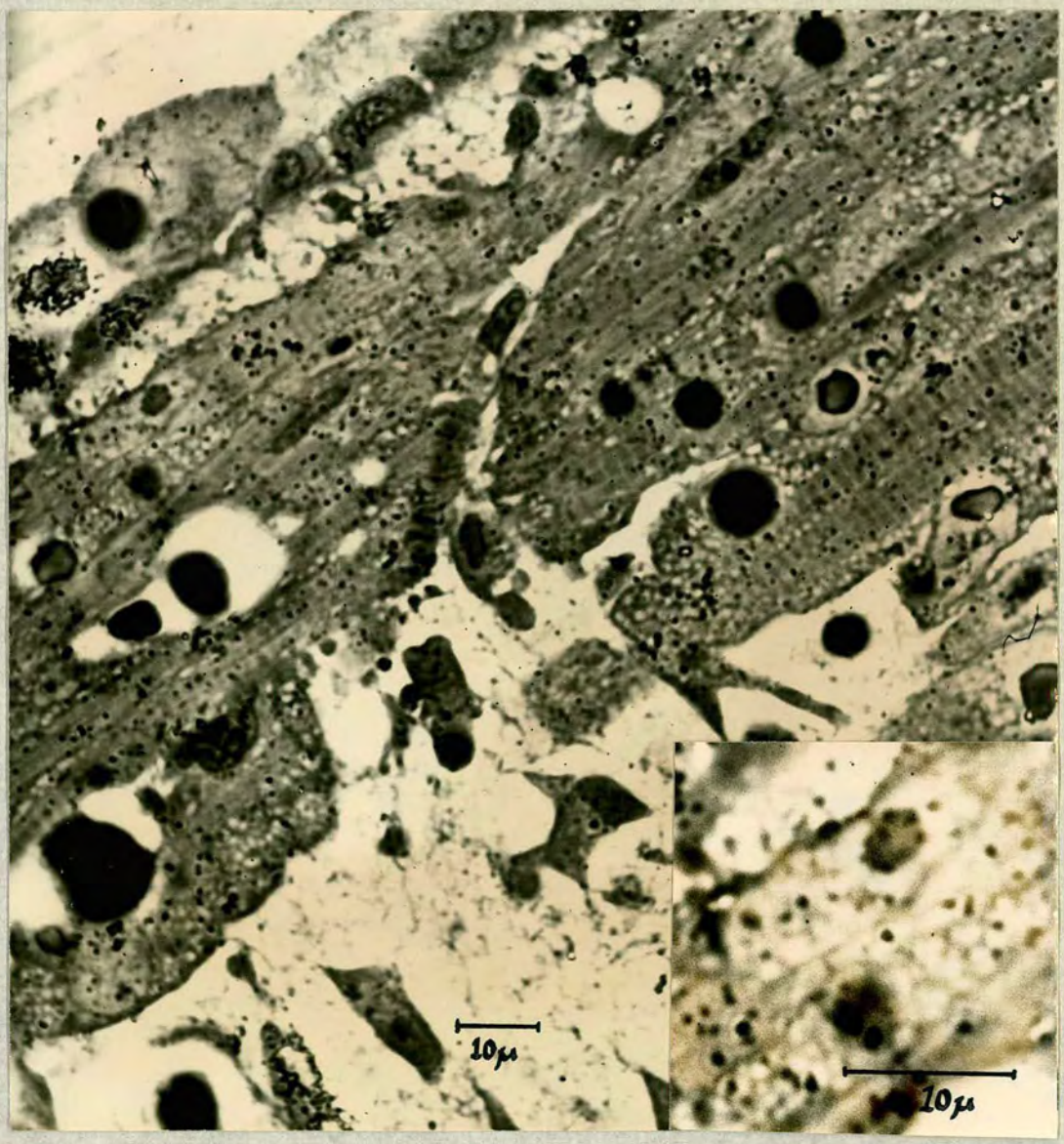


PLATE (5). 1 hour pulse of 3H-T. Inset shows labelled nucleoli. 4 weeks exposure. Myofibrils are labelled. Non-myofibrillar sarcoplasm is lightly labelled. Non-muscle cytoplasm is not labelled.

PLATE (6). 6 hour pulse of 3H-T. 4 weeks exposure. Similar labelling pattern to plate (5). Myofibrils are more heavily labelled.

A: Electron-Miscrope Autoradiograms

These were coated at a time when I had not perfected the technique and were of a very poor quality. Only in a very few instances can any cell structures be seen through the thick emulsion. Because of the length of time required to cut the sections and expose the autoradiograms they have not yet been repeated. One EM plate (Plate (1)) is shown to make the point that many of the silver grains in the fibrillar regions of muscle cells are actually located over the myofibrils and are distinct from the grains over mitochondria, confirming the finding of Jones (see introduction). This plate happens to be the result of a 6 hour pulse followed by a 6 hour chase.

B: Light Microscope Autoradiograms

(exposed for 4 weeks unless stated).

(i) Controls: Plates (2) and (3) show the effect of TCA on 12 hour pulsed material. Plate (3) shows material which after glutaraldehyde fixation was incubated in MES/Mg buffer at 37^{o} for 30 minutes (as part of the DNAse control) and then washed in TCA to remove any non-incorporated 3HT which may be present. Plate (2) shows material which was merely rinsed in phosphate buffer, as were all of the other pieces, after glutaraldehyde fixation. The autoradiographic

exposure times and magnifications are the same. The concentrations of silver grains over myofibrillar cytoplasm and labelled nuclei are not noticably different. So most, if not all of the silver grains in the autoradiograms represent a derivative of thymidine which is not soluble in TCA and hence is probably of high molecular weight.

Material treated with DNAse was as heavily labelled as material not treated with DNAse. Either the high molecular weight material is not DNA or the enzyme treatment is not effective. A similar problem was found when RNAse is used on material labelled with 3H-uridine (Chapter 3.3) and it was concluded that probably the enzyme was ineffective in these conditions.

AMD; Material incubated with 3HT for 12 hours in the presence of the RNA transcription inhibitor AMD still shows heavy labelling over muscle cell cytoplasm. This is to be expected if the 3HT uptake is into DNA rather than RNA. Tritiated uridine uptake is abolished in these conditions (Chapter 3.4). It is concluded that the labelling may represent newly formed DNA in that it is derived from ^{3}H-methyl thymidine, it is insensitive to AMD and it is insoluble in TCA, but that conclusive proof that the labelling represents

DNA awaits further investigation. For instance DNAse treatment could be attempted on sections of the labelled tissue embedded in the water miscible medium glycol methacrylate, or DNA and RNA could be extracted from the labelled material after separating nuclei from the cytoplasm, and the distribution of tritium in the two substances could be measured. A further test will be provided by an experiment being performed by K.W.Jones et al in this laboratory using the technique of in situ hybridization of nucleic acids (John et al 1969 and Gall & Pardue 1969). 26S myosin mRNA (see Chapter 3.1) was extracted from tritium labelled muscle tissue and hybridized onto cytological preparations of muscle tissue. This will specifically label sites in the cell containing DNA having a complementary sequence to that of the mRNA.

(ii) Pulse label experiments.

A 10 minute pulse with 3HT gives the result shown in Plate (4). The only heavy labelling is over some nuclei which do not appear to be in myotubes. Here the labelling is intense and uniform. There is very light labelling over myofibrils. Elsewhere, i.e. over interfibrillar sarcoplasm, muscle nuclei, many non-muscle nuclei and non-muscle cytoplasm there is no significant labelling.

A 1 hour pulse with 3HT, as shown in Plate (5) gives the same result in non-muscle cells as a 10 minute pulse, i.e. a few nuclei are densely labelled and the rest of the nuclei as well as the cytoplasm show no significant labelling. There are some instances of muscle nuclei becoming lightly labelled, particularly at the nucleolus (arrows). Muscle cytoplasm is more heavily labelled than in the 10 minute pulse. A 6 hour pulse, as shown in Plate (6), gives the same result as the 1 hour pulse but with a higher grain density. Non-muscle cytoplasm is still not labelled, as can be seen in epidermal and presumably neural tissues in the plate. Muscle nuclei have an intermediate grain density between the two extremes of labelling found in non-muscle nuclei, i.e. they possess a significant number of grains but are not as densely labelled as nuclei presumably about to enter mitosis. There is no apparent difference here between the density of grains over nucleoli and over nucleoplasm. The cytoplasmic grains of muscle cells here appear to be more numerous over myofibrils than over interfibrillar regions. Pulsing for 12 hours probably generally increases the level of labelling where labelling already exists whilst maintaining the same overall pattern (Plates (2) and (3)).

(iii) Chase experiments

Chases of 1 and 6 hours after pulses of both 1 and 6 hours give no apparent changes in grain density over muscle nuclei or cytoplasm. Chasing does however have an effect on 10 minute pulsed tissue. After 29-33 weeks exposure 10 minute pulsed myofibrils show a very distinct heavy label, and the muscle nuclei are black with silver grains. After a 1 hour chase there is no apparent change but after a 6 hour chase nuclei are still black with silver grains whereas myofibrillar labelling has disappeared.

4.4. DISCUSSION

No definite conclusions can be reached until it is conclusively demonstrated that the thymidine labelling observed in the autoradiograms represents DNA. The case is not so strong as that for uridine labelling in myofibrils representing RNA (Chapter 3) where polysomes known to contain RNA have been observed in association with myofibrils.

In the case of non-mitochondrial DNA there is no independent work to suggest what might be its cytological appearance. The types of controls necessary are described in the results section.

The cytoplasmic labelling may be artefactual in that the incubation conditions may cause nuclear damage and release of nuclear DNA into the cytoplasm. This DNA may then fortuitously associate with myofibrils. This possibility has not been directly tested but two observations make this seem unlikely. First, only muscle cell cytoplasm becomes labelled, and all muscle cells observed become labelled. Unless muscle cell nuclei uniformly respond differently to non-muscle nuclei during the incubation, it is unlikely that the cytoplasmic labelling is an artefact due to nuclear radiation damage. That muscle nuclei probably are not differentially damaged is supported by the second observation, that no evidence of nuclear damage was seen in any cells incubated in medium of the same specific activity in tritiated uridine for up to 15 hours (see Chapter 2, e.g. plates 1 & 17), when viewed electron microscopically. Nuclear damage, then, seems unlikely.

Independent evidence from studies on dystrophic mouse muscle (Appendix 7.1) also suggest that DNA may be closely associated with myosin. If this labelling does represent DNA what are its properties and possible functions? The DNA would be specifically found in muscle tissue cytoplasm, certainly with little

if any in epidermal or neural tissue. It would be rapidly synthesized (it labels rapidly) and non-mitochondrial. It would be stable for at least 1 hour but may not be stable for as long as 6 hours, as a 6 hour chase after a 10 minute pulse removes cytoplasmic label, although it does not if the pulse lasts for 1 hour before the chase. As the label appears in the cytoplasm in the shortest pulse time (10 minutes) and because chasing apparently does not increase the specific activity of the cytoplasm or decrease that of the nuclei, the DNA would probably be synthesized in the cytoplasm. The DNA would then become preferentially located in the myofibrils. A highly speculative role for this DNA is that of determinator of muscle differentiation, i.e. a self replicating cytoplasmic gene giving the cell the exclusive ability to differentiate into a muscle cell. In tissue culture, mononuclear myoblasts do appear to remain determined as myobasts even when cultured through many cell generations before being allowed to reach confluence and differentiate into myotubes. This is shown in the behaviour of myogenic cell lines (Yaffe 1968) although there is nothing in these observations to say whether the determinator is in the nucleus or the cytoplasm. If it is in the cytoplasm it would have to be self replicating to explain the persistence

of determination through many cell generations. This role could be performed by self replicating cytoplasmic DNA.

Another possibility is that this cytoplasmic gene could be the product of reverse transcriptase, previously referred to, using mRNA as a template. The possible role of this rapidly labelling cytoplasmic DNA is discussed in a general discussion in Chapter 7.2.

4.5. SUMMARY

Evidence is presented for non-mitochondrial uptake of tritiated thymidine into a TCA insoluble substance in the cytoplasm of muscle cells and not of other tissues. This substance becomes associated with myofibrils and is thought to be DNA. The significance of this finding is discussed.

CHAPTER FIVE

CYTOLOGICAL LOCALIZATION OF POLY-A SEQUENCES IN XENOPUS TISSUES AND CULTURED MUSCLE CELLS

5.1. INTRODUCTION

There is a growing body of evidence that eukaryotic mRNA molecules have a polyadenylic acid sequence (poly-A) at the 3' end, and speculation that this segment may somehow protect the message from the intranuclear degradation that appears to be the fate of the bulk of the nuclear non-ribosomal RNA (Chapter 3.1).

When RNA is extracted from rat liver membrane bound microsomes (biochemical word for ribosomes) with

phenol in the usual neutral pH buffers, the RNA is mainly rRNA. However, if the phenol is re-extracted with pH9 buffer an RNA containing an unusually high amount of adenine (50-60 moles% of AMP) is found. The buoyant density of this RNA is about 10S which is in the right region for monocistronic messages for most proteins (Hadjivassiliou & Brawerman 1966). The buoyant density peak sharpens when the RNA has been treated with pancreatic RNAse, to which poly-A is resistant, and indeed the product is now even more enriched in adenine. These findings were confirmed in Ehrlich ascites cells (Edmonds & Caramela 1969) who further purified the poly-A rich species from the pH9 extraction by chromatography on a poly-dT-cellulose column, and estimated that 1% of RNA synthesized by these cells in 12 hours consisted of adenine-rich RNA. They also found that the poly-A rich RNA was found predominantly in the nucleus and that its synthesis was sensitive to fairly high doses of AMD. Darnell et al (1971a, b)looked for poly-A sequences specifically in heterogeneous nuclear RNA (HnRNA), (see Chapter 3.1) and polysomal mRNA in HeLa cells by blocking rRNA synthesis with low doses of AMD. They found that HnRNA and mRNA both contain poly-A sequences (characterized as RNAse resistant fractions appearing as labelled regions in electrophoresed polyacrylamide gels, taking up adenosine label but

not uridine label),and that label appears in HnRNA before it appears in mRNA. Higher levels of AMD almost totally block even HnRNA and mRNA synthesis whilst only partly reducing labelled poly-A yields, indicating that its synthesis is independent of that of the molecules to which it becomes attached and is probably independent of a DNA template. This view is supported by the action of the adenosine analogue cordycepin (3'd adenosine) which greatly reduces synthesis of poly-A but only slightly reduces total HnRNA and mRNA synthesis. Polysomal mRNA and HnRNA were then hybridized to DNA under conditions which would allow only highly reiterated sequences to form DNA-RNA duplex molecules. Previous work by the same author has shown that each HnRNA and mRNA molecule has a stretch whose sequence is reiterated on the DNA molecule. Only the duplex molecules from the mRNA hybridization are enriched in poly-A. The less reiterated parts of the mRNA molecules were not enriched in adenosine and the HnRNA was as enriched in adenosine whether reiterated or not. This shows that mRNA contains a poly-A sequence which is either transcribed from the DNA or is added to the molecule after transcription adjacent to a reiterated sequence unit. The significance of the findings with HnRNA is not clear. The authors put forward the hypothesis that the poly-A

sequence protects from degradation the few HnRNA molecules destined to become mRNA. The existence of poly-A sequences in both HnRNA and mRNA molecules in HeLa cells was confirmed by Edmonds et al (1971) using the poly dT cellulose column technique. Poly-A was found to selectively bind to nitrocellulose (Millipore filters) and this technique, together with the RNAse and uridine labelling tests, was used to show the presence of poly-A sequences in mouse sarcoma 180 ascites cells (Lee et al 1971) which was further characterized as being a stretch of about 200 nucleotides in length composed of 97-99 moles% adenylate and 0.5% adenosine, showing the 3' and also to be adenine based. Labelling kinetics show that this segment is added after the synthesis of the rest of the molecule. The nuclear RNA poly-A segments were longer than those found in polysomal mRNA. Poly-A sequences have also been found in haemoglobin mRNA (Burr & Lingrel 1971; Lim & Canellakis 1970; Pemberton & Baglioni 1972). RNA virus genomes (Lai & Duesberg 1972; Green & Cartras 1972) and DNA virus specific RNA in infected cells (Philipson et al 1971). Reports have not yet appeared concerning poly-A sequences in mRNAs for the myofibrillar proteins.

Elsewhere in this thesis it has been suggested that the mRNAs for myofibrillar proteins become attached

to the myofibrils and that the fibrous region of the nucleolus and anucleolar blobs may be concerned with processing mRNA. It would be interesting then to cytologically locate poly-A sequences in muscle cells. As the only cytoplasmic RNA containing poly-A appears to be mRNA, the presence of poly-A in myofibrils would indicate that mRNA is associated with myofibrils. The presence of poly-A in the nucleolus or blob would support, but not prove the view that mRNA processing is taking place there. Within the nucleus it is not clear if any RNA not destined to become cytoplasmic mRNA contains poly-A sequences.

Preliminary experiments have been performed to locate poly-A cytologically, using the technique of in situ hybridization of nucleic acids (John et al 1969, Gall & Pardue 1969). The tissue is fixed and any double stranded nucleic acids denatured by heat, acid or alkali. Radioactive nucleic acid, e.g. a specific type of RNA, is applied under suitable temperature, pH and ionic conditions for specific hybridization to take place. The radioactive RNA will then form a sequence specific hybrid with denatured complementary DNA present in the tissue. Non hybridized (single stranded) radioactive RNA is then removed with RNAse (double stranded molecules are resistant to RNAse)

and autoradiograms are prepared. In the present experiments the radioactive nucleic acid is tritiated polyuridylic acid (3H-poly-U) which will specifically hybridize with poly-A in the specimen. Jones (unpublished results in this laboratory) used this technique with mouse L cells and found a high concentration of poly-A in the nucleoli, with some in the rest of the nucleus and the cytoplasm. Here the technique is used on cells of a Xenopus cell line, a rat myogenic cell line, and on anucleolar and wild-type Xenopus embryo squashes.

5.2. MATERIALS AND METHODS

The Cells:-

'Xenopus cells' are a cell line maintained in this laboratory derived by Rafferty (unpublished) from wild-type Xenopus laevis kidneys. They have an epithelial morphology. They are used when actively growing and semi-confluent. Rat myoblasts are a cell line derived from clone 511552196 maintained in this laboratory by Finn Guinness and originally from Dr David Yaffe, Weizmann Institute of Science, Rehovot, Israel.

Rat myoblasts are allowed to grow to confluence, fuse and differentiate into multinucleate contractile myotubes.

Xenopus embryos are 1 week post-hatched embryos (stage 21-22 Rugh 1948) from a 1-nu x 1-nu cross as in Chapter 3.2. They were sorted by eye into wild-type and 0-nu phenotypes, the 0-nu having oedematous head and gut, microcephali, microphthalmia and bent tail tips.

Culture media and conditions:-
Xenopus cell medium was 1x Eagles Minimal Essential Medium, Banks based + non-essential amino acids (Wellcome) + 10% foetal bovine serum (Flow laboratories), + 40μg/ml streptomycin sulphate (Glaxo) + 100μg/ml benzyl penicillin (Glaxo) + 1.1% sodium bicarbonate. Cells are incubated at 28° in 95%O_2 in 85mm Falcon plastic petri dishes.

Rat myoblast and myotube medium was 1xMEM + 10% foetal bovine serum (each batch tested for ability to support fusion and differentiation of myoblasts) + 10μg/ml kanamycin. Cells are incubated at 37° in 95%O_2, 5%CO_2 in 85mm Falcon plastic petri dishes.

Xenopus were grown in tap water at room temperature (about 20°).

In situ hybridization:-
The tissue culture cells were rinsed in Dulbecco A solution (a physiological saline made from Oxoid

tablets) to remove serum and antibiotics and fixed on the dishes for 30 minutes at 0^{o} in 3 volumes of methanol + 1 volume of glacial acetic acid, changed at 10 minute intervals. The embryos were transferred directly from water to fixative and the somite regions dissected out. The embryo pieces were then placed in 45% acetic acid and squashed by hand on gelatinized slides (see Chapter 3.2) under glass coverslips coated in silicone by dipping in a solution of 2% dimethyldichlorosilane in carbon tetrachloride (Repelcote, Hopkins and Williams Ltd.) and baking at 65^{o} for 1 hour. The coverslips were then removed with a scalpel after freezing the whole preparation solid on a piece of solid CO_2. The Xenopus squashes were then dehydrated through 70, 90% and absolute ethanol and air dried and stored. Tissue culture cells were rehydrated through 100, 90, 70 and 50% alcohol and distilled water immediately after fixation, at RT.

All preparations were acid denatured by placing in 0.2N HCl pH 0.8 for 20 minutes at RT. They were then dehydrated through the alcohol series and dried under vacuum. The tritiated polyuridylic acid (3Hpoly-U) is 20μg/ml, 0.5×10^{6} cpm/μg in 0.3M NaCl, 0.01M sodium acetate and was synthesized by Dr John Bishop of this laboratory. The poly-U solution was applied to the denatured,

dehydrated cells by applying a drop containing about 5µl to the cells under a clean 18x18mm coverslip, the edges of which were sealed with a solution of rubber in petroleum ether. Batch 1 poly-U was pH5.5 and this was used with xenopus cells and rat myoblasts and myotubes. Batch 2 poly-U was pH 7.5 and was used on Xenopus squashes and again with Xenopus cells. Experiments with batch 2 poly-U gave a very low level of labelling after prolonged autoradiographic exposure and these results are not presented. All the photographs in the results section are with batch 1 poly-U.

Hybridization was performed by floating the preparations in aluminium foil trays in covered water baths at 30° for 4.5 hours. The coverslips and rubber were then removed and the preparations were briefly washed in 2xSSC (0.3M NaCl+0.03M sodium citrate pH 7.2) at 0° and RNAsed in 20µg/ml RNAse A (5x crystallized bovine pancrease, Sigma) in 2xSSC at 0° for 20 minutes, (the stock 10x solution of RNAse was previously boiled for 10 minutes and then cooled to remove possible traces of DNAse activity). The preparations were then washed in a large volume (approximately 11/15 coverslip preparations) of 2xSSC at 4° for 2 hours, continuously stirred. They were then dehydrated in alcohols and then air and vacuum dried. Duplicate experiments were performed with the Xenopus cells and the muscle cells

in which the poly-U was made approximately 2μg/ml in RNAse, stood at RT for 10 minutes (as a droplet on a siliconized coverslip in a moist atmosphere) and then used in place of untreated poly-U.

Autoradiography:-

The slides or strips of petri dish were coated with photographic emulsion (K2 Nuclear Research Emulsion, Ilford), exposed, developed and fixed as described for thick section autoradiograms in Chapter 3.2.

Cytology:-

The autoradiograms, usually still wet, were stained in either 0.02% w/v toluidine blue (Gurr) in distilled water for 30-60 minutes at RT or Giemsa stain (1.5ml Giemsa R66 (Gurr) + 50ml pH6.8 buffer made by dissolving one Giemsa buffer tablet (Gurr) in 50 ml distilled water) for 1½ hours at RT, briefly rinsed in distilled water and air dried. They were then mounted in Zeiss immersion oil under a coverslip and photographed on a Zeiss microscope using x100 oil immersion and x40 objectives.

5.3. RESULTS

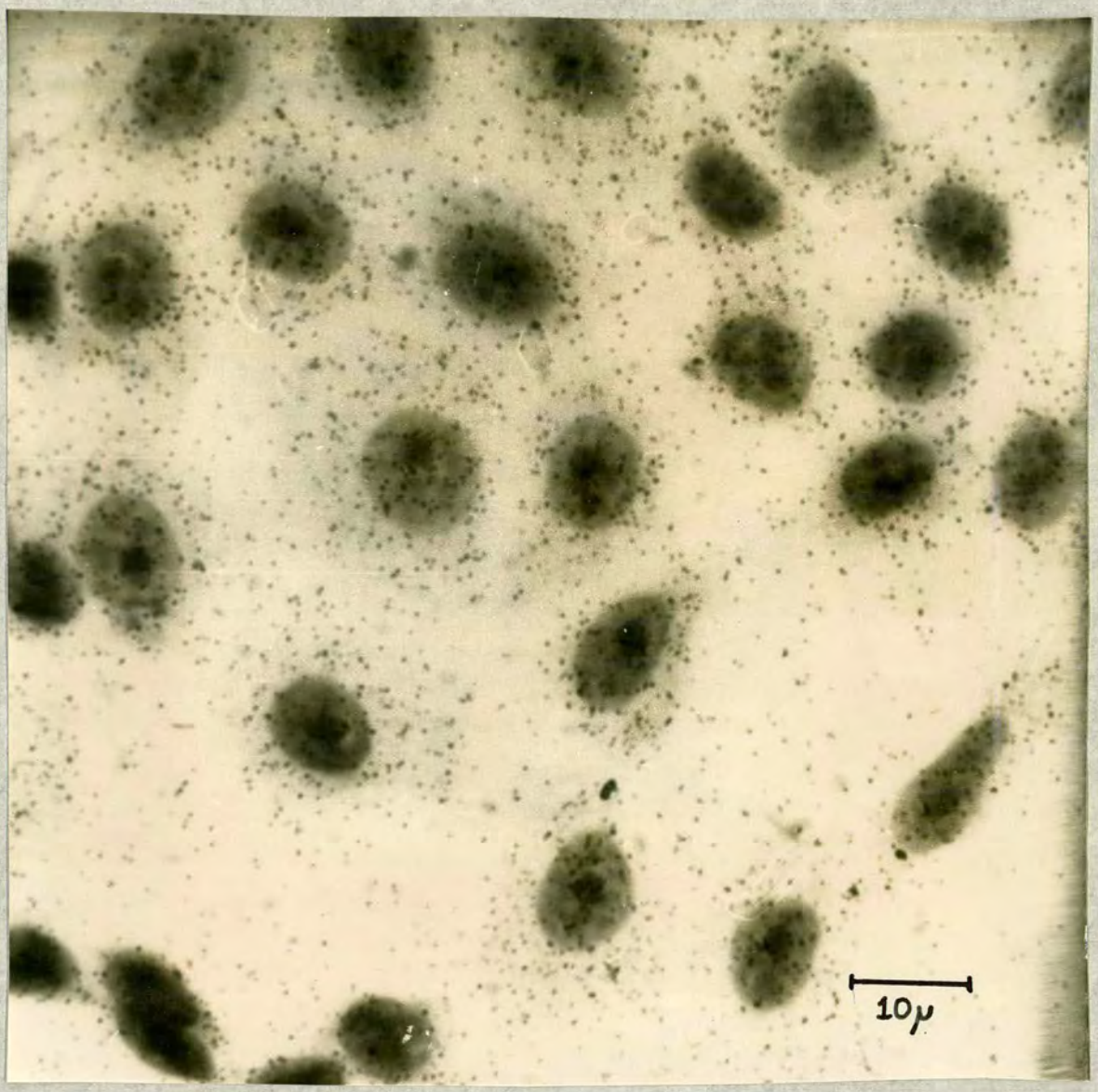


PLATE (1). Xenopus cells. Poly-U. 73 days exposure. Stained in toluidine blue.

Nuclei, nucleoli and cytoplasm (faintly stained) are labelled. Experimental details in text.

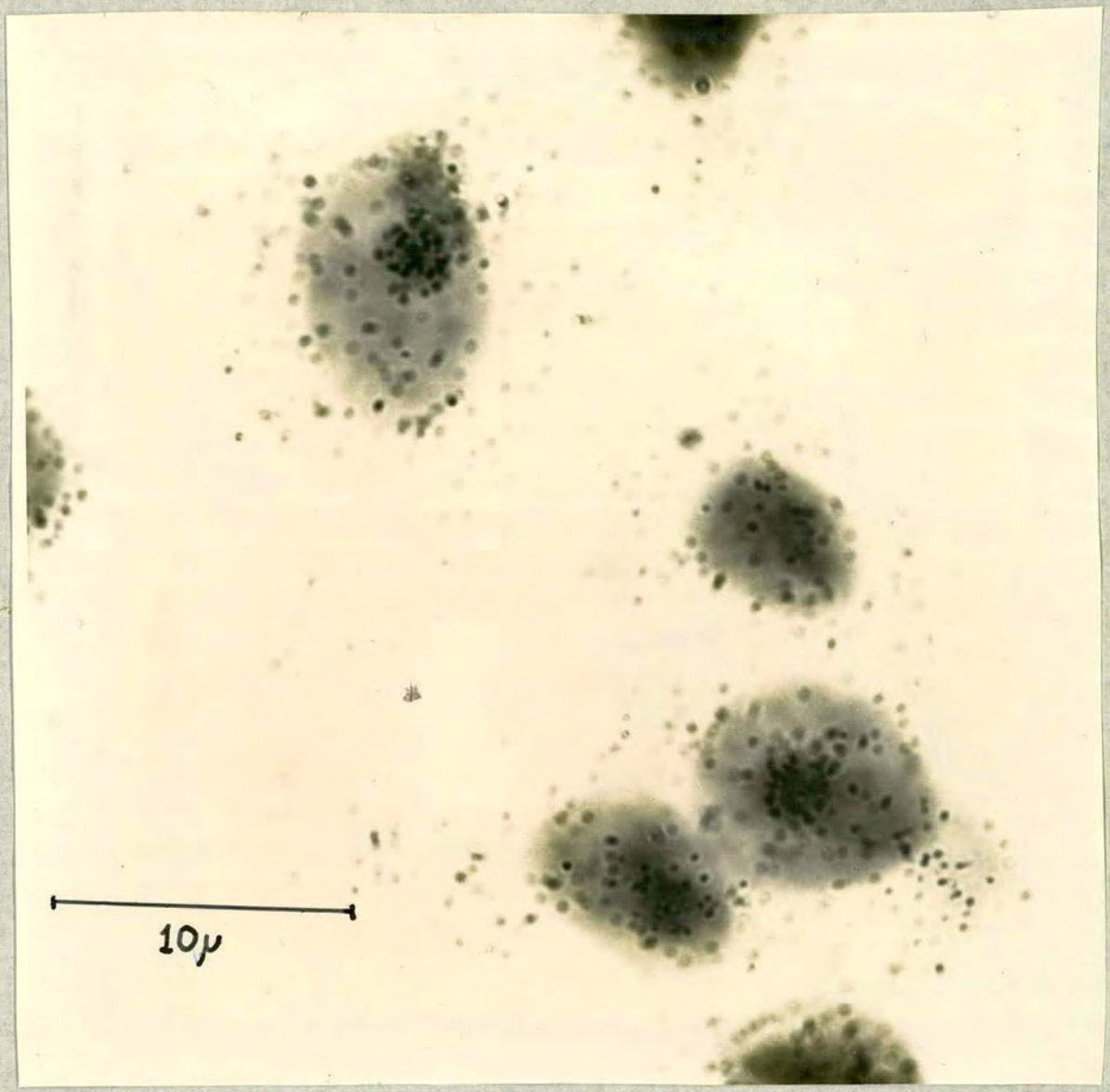


PLATE (2). Xenopus cells, as Plate (1), showing that some nucleoli are preferentially labelled.

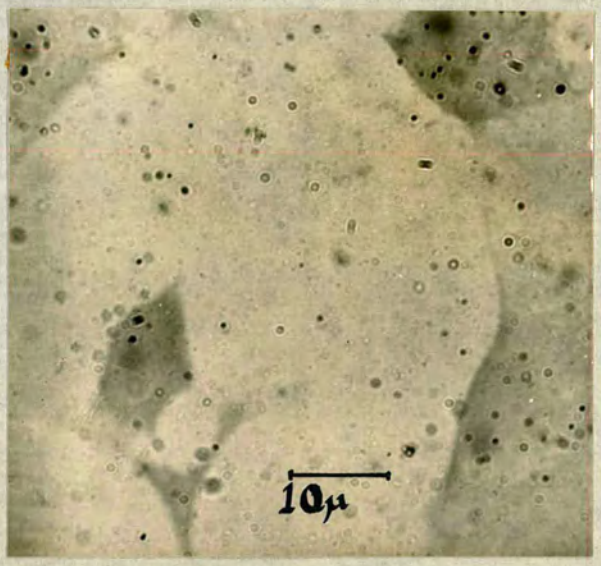


PLATE (3). Xenopus cells. Same as Plate (1) but incubated with RNAse-degraded poly-U (see text). Very few silver grains are present.

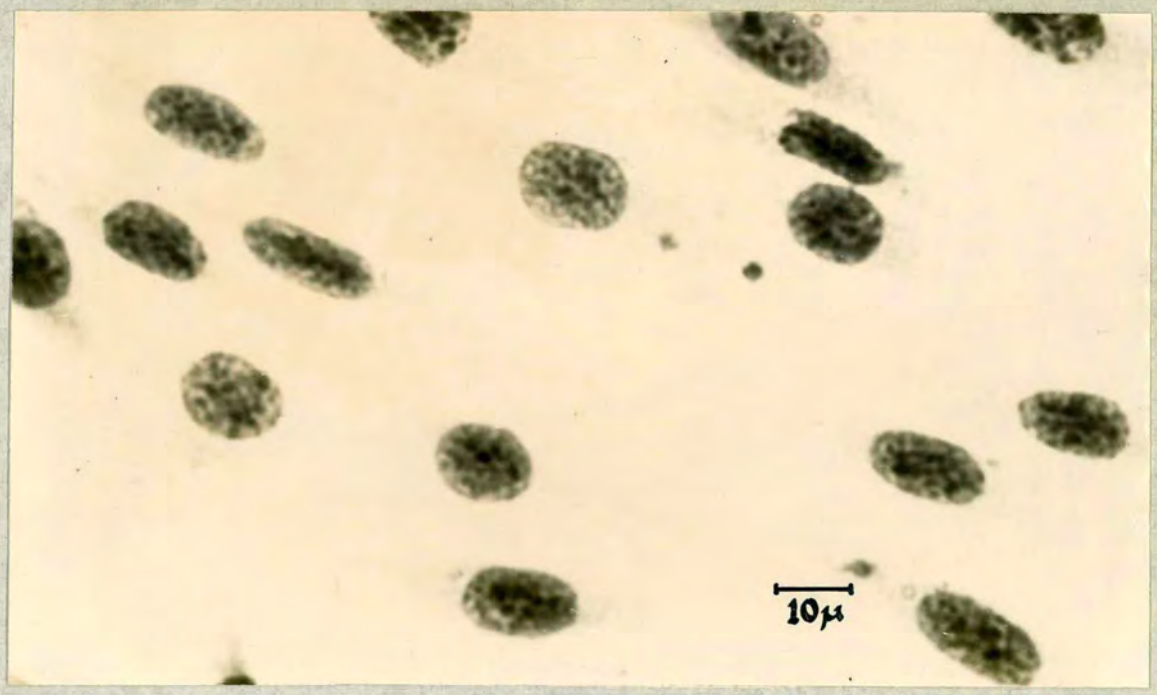


PLATE (4). Mononuclear rat myoblasts, treated with poly-U and RNAsed (see text). 70 days exposure. Stained with toluidine blue. No silver grains are present.

PLATES 5, 6 and 7. Mononuclear rat myoblasts treated with poly-U (see text). 70 days exposure. Stained with toluidine blue.

PLATE (5). A nucleus with a heavily labelled nucleolus.

PLATES (6 and 7). Heavy labelling of small areas of cytoplasm.

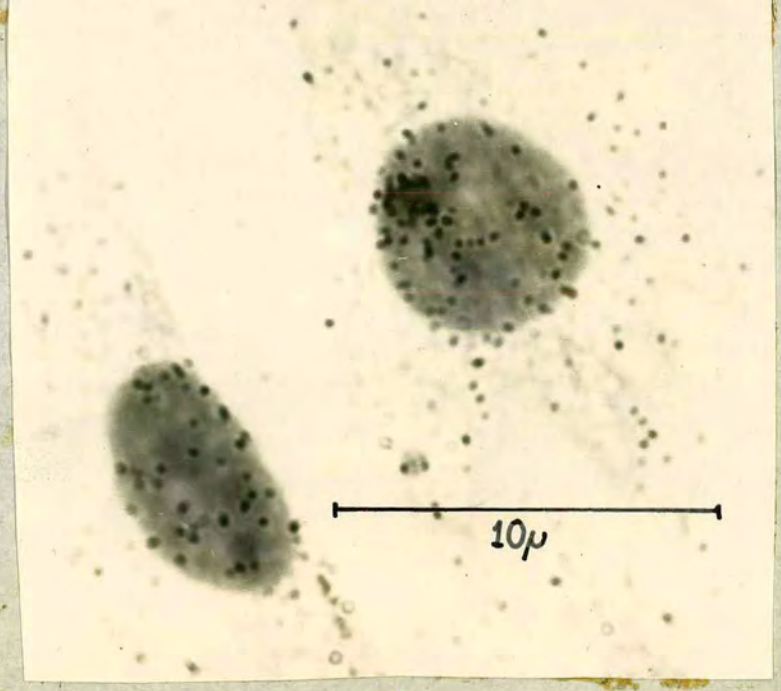


PLATE (5)

PLATE (6)

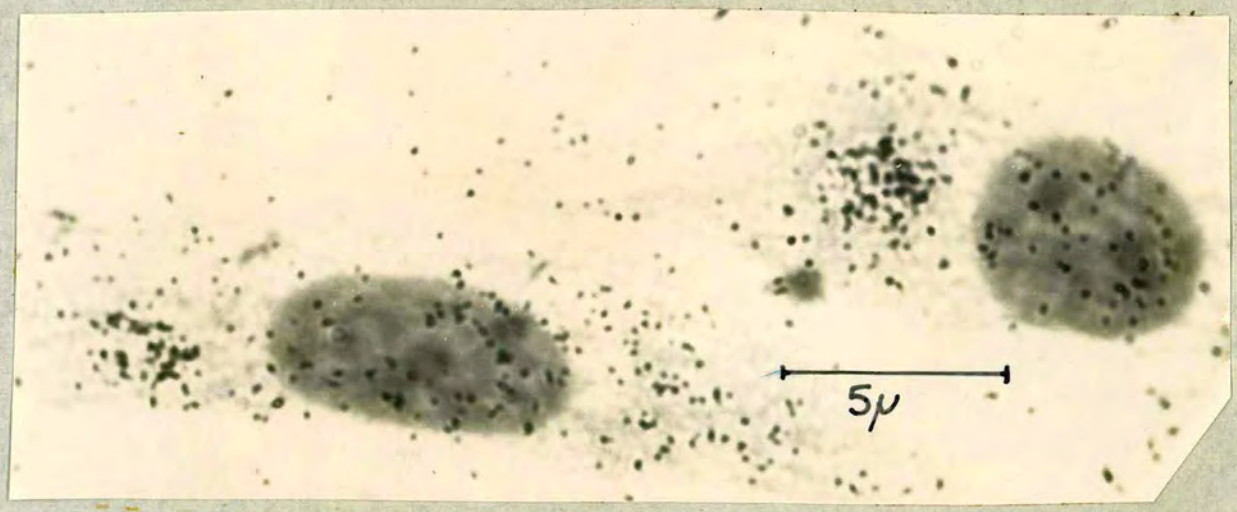
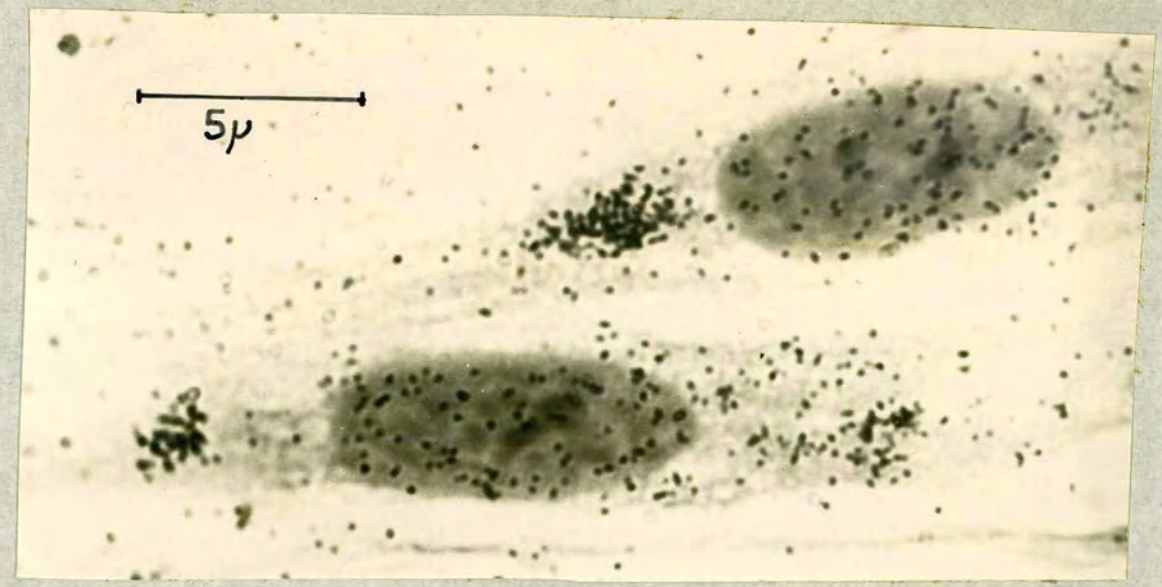


PLATE (7)



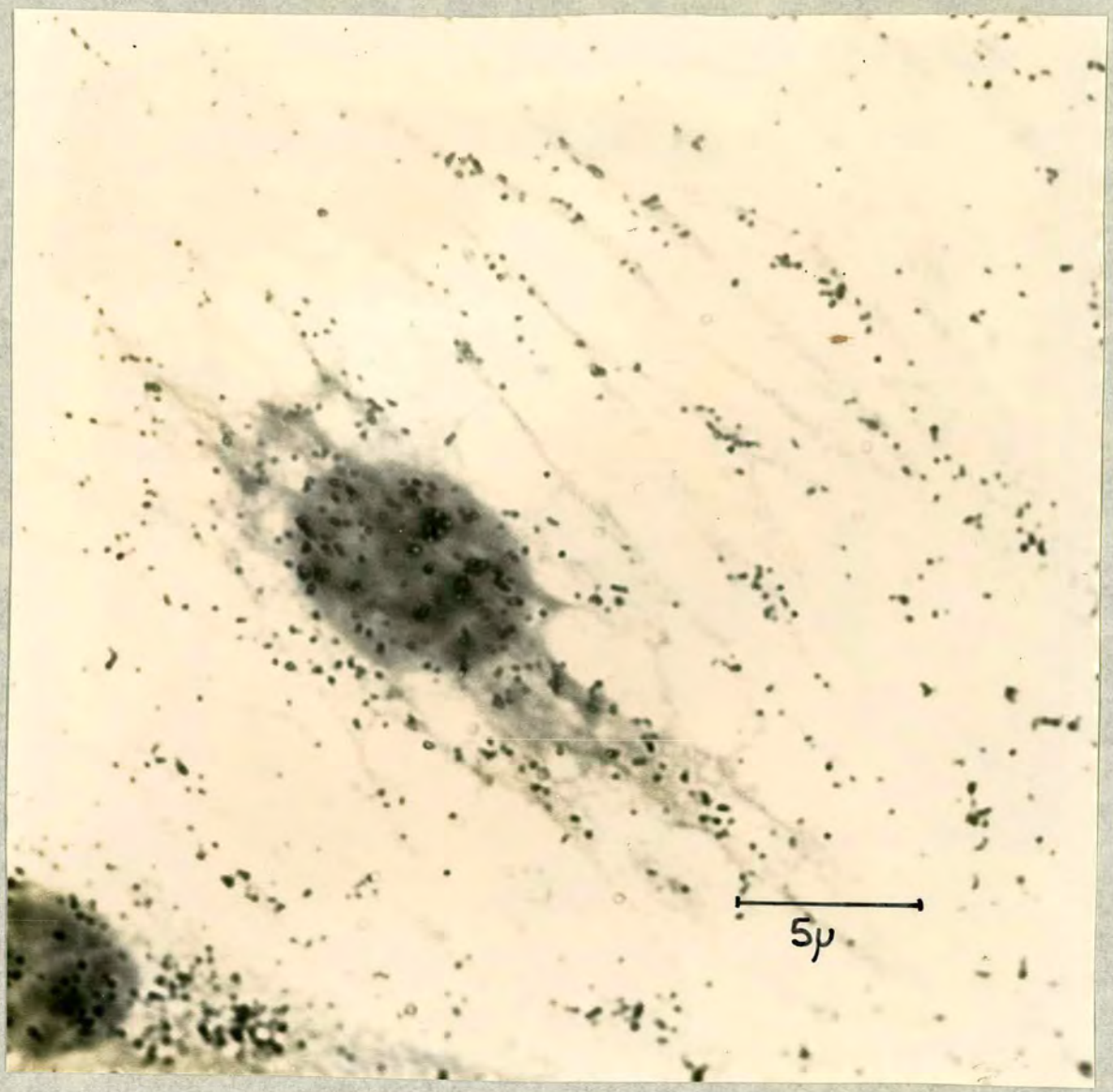


PLATE (8). Mononuclear rat myoblast as in Plates 5, 6 and 7. The cytoplasm is thinly spread and contains darkly staining strands which are longitudinally orientated in the cell, all of the cytoplasmic labelling appears to be in these strands.

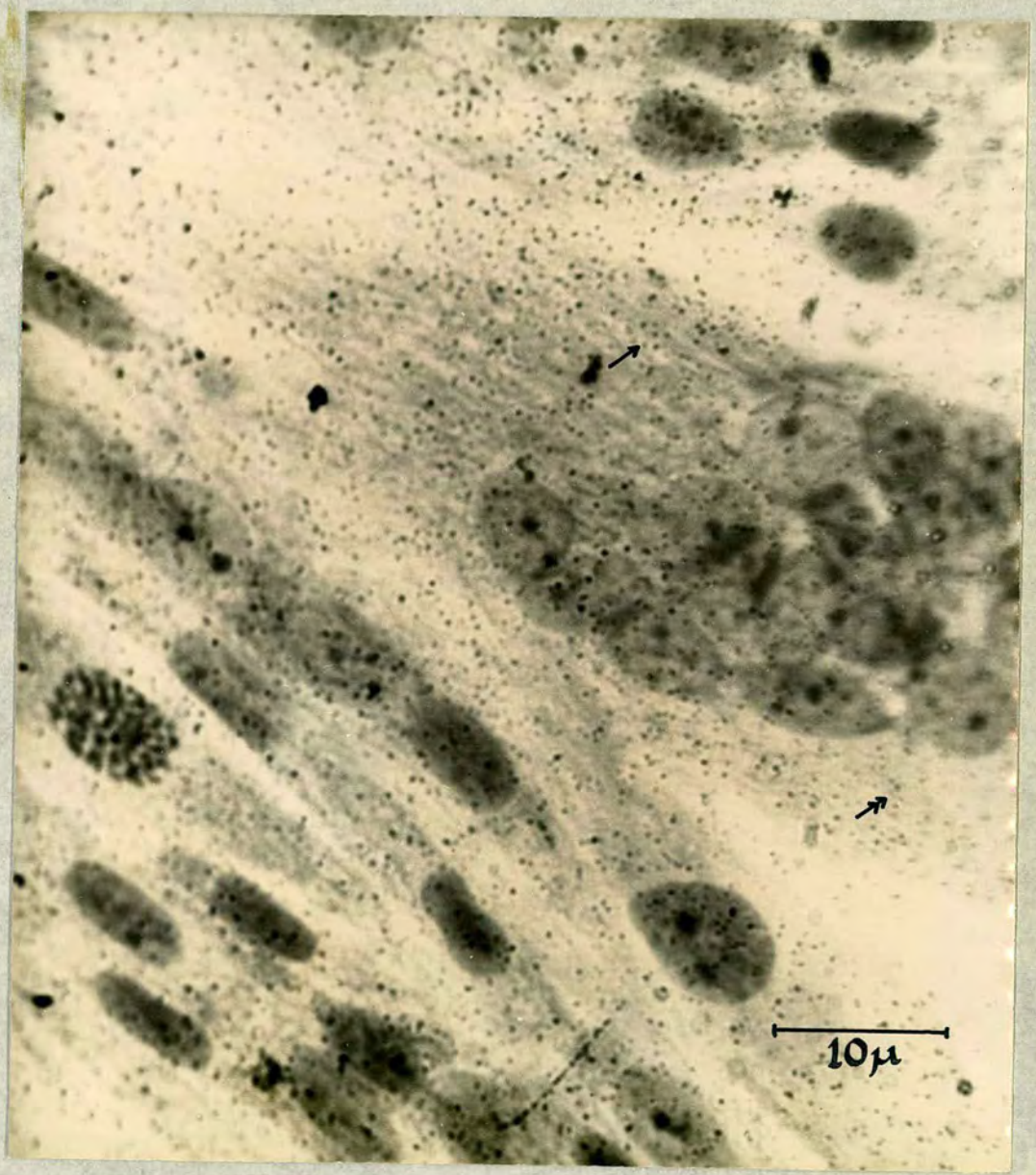


PLATE (9). Rat myotubes. Poly-U. 70 days exposure. Stained with toluidine blue. Nuclei and cytoplasm, including myofibril-rich region (⟶) are labelled. A region of cytoplasm with no visible myofibrils is labelled (⟶⟶). Experimental details in text.

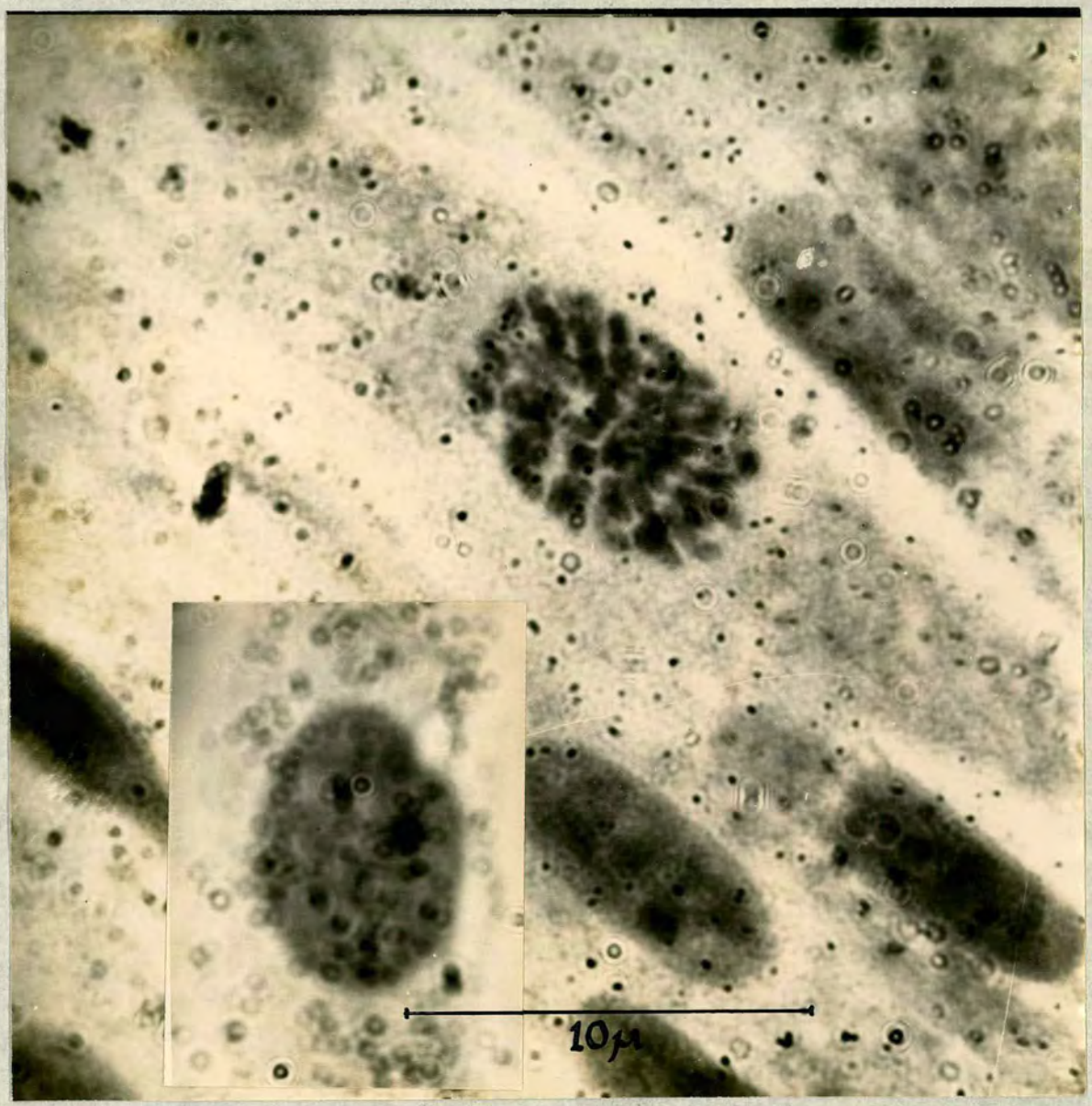


PLATE (10). Rat myoblasts and myotubes, showing a myoblast undergoing mitosis. Inset is an interphase rat myoblast nucleus. The interphase nucleus is more heavily labelled than the mitotic nucleus.

Poly-U. 70 days exposure. Stained with toluidine blue. Experimental details in text.

Xenopus cells:-

Plate (1) shows heavy labelling in the nucleus, nucleolus and cytoplasm. In many cases the whole cell is uniformly labelled. However, Plate (2) shows some cases of distinctly heavier labelling in the nucleolus than the rest of the cell. That this is not the case in every cell could be due to different nucleoli having different specific activities or to all nucleoli having the same specific activity but many of them lying too far below the surface of the cell and the photographic emulsion to produce the maximum number of silver grains. If the nucleoli do have different specific activities then it would appear that poly-A only associates with the nucleolus at certain times during interphase, and not for the whole of interphase. The data is not able to distinguish between the two possibilities.

That all or most of this labelling is due to binding of 3H-poly-U by cellular components is shown by the fact that treating the poly-U with RNAse before and during hybridization removes most of the subsequent labelling (Plate 3).

Rat muscle cells:-

In this cell line striations are not seen in myofibrils even in contracting myotubes; the myofibrils are

less well organized than in Xenopus somites. Some densely staining structures are seen in mononuclear cells before fusion (Plates 5-7) but it is not clear if these are myofibrils. Myofibrils appear more clearly in multinucleate myotubes (Plate 9).

Labelling in mononuclear cells after poly-U hybridization is distinctly patchy (Plates 5-8). Sometimes the nucleus is more heavily labelled than the cytoplasm (Plate 5), showing that in these cells the nucleus contains more available poly-A molecules than the rest of the cell. Occasionally nucleoli are seen to be more heavily labelled than the rest of the nucleus (Plates 5 & 8) but usually this is not so (Plates 6 & 7). As with Xenopus cells this could be due to equal specific activity nucleoli lying at different depths below the photographic emulsion or to a genuine difference in specific activities of nucleoli in different cells. The latter explanation is favoured in this case because of the much lower frequency of heavily labelled nucleoli here than in Xenopus cells, although this could be due to the two cell types having different shapes. If true, however, this observation implies that poly-A associates with mononuclear rat myoblast nucleoli at restricted times in development. Heavily laballed

nucleoli were not seen in rat myotubes (Plate 9), showing that poly-A does not markedly associate with nucleoli after fusion.

An exciting discovery was the observation of well circumscribed, heavily labelled regions of the cytoplasm of mononuclear cells (Plates 6 and 7). At the light microscope level there seems to be nothing special about the cytology of these regions. They are usually close to the nucleus and 'in line' with the nucleus rather than alongside it; i.e. in these elongated cells the nucleus and the heavily labelled regions are both on the long axis of symmetry. The only organelle which tallies for size, number and position is the Golgi apparatus (see refs in Chapter 2.1). However a definite identification of this labelled structure awaits EM autoradiographic data. Such densely labelled regions of cytoplasm were not observed in any of the other cell types examined. It may be that they are specific to an early stage of myogenesis.

Again, treatment of the 3H-poly-U with RNAse before and during hybridization abolishes labelling of mononuclear rat myoblasts (Plate 4) and myotubes, showing that labelling probably represents the presence of

poly-A sequences in the cells. Myotubes containing many nuclei and densely staining longitudinally orientated but non-striated myofibrils again have a fairly uniform covering of silver grains over nuclei and cytoplasm, including myofibrillar areas. These myofibrils are not so well demarcated as in sections of embryonic tissues and so it is not possible to say if there are more grains over myofibrils than over non-myofibrillar cytoplasm. However, the arrowed region indicates an area of sarcoplasm obviously containing a high proportion of myofibrillar material to non-myofibrillar material and there is certainly no less labelling than in an area with less fibrillar material (double arrow) or in the cytoplasm of a mononuclear cell (Plate 9). As the presence of myofibrils does not reduce labelling, the myofibrils probably are labelled. Again in myotubes the addition of RNAse to the 3H-poly-U drastically reduces the labelling.

Myofibrillogenesis is often seen to precede fusion in myoblasts (refs in Chapter 2.1). Plate (8) shows a mononuclear myoblast with longitudinally orientated, densely staining strands in the cytoplasm which are probably myofibrils. These structures are heavily labelled whereas the intervening cytoplasm does not

appear to be labelled at all. This, then is evidence that myofibrils contain poly-A sequences and hence probably mRNA.

In one of the myotube preparations a cell, presumably a mononuclear myoblast not yet fused, was observed undergoing mitosis (Plates 9 and 10). Although the chromosomes (or surrounding nuclear sap or cytoplasm) are as heavily labelled as some of the less heavily labelled nuclei in myotubes (same plates) there are certainly far fewer silver grains over them than over the nucleus of a typical mononuclear myoblast in interphase (inset in Plate 10).

Thus poly-A synthesis appears to take place during interphase and cease at mitosis, in common with most other species of RNA. The results do not exclude the possibility that a template for poly-A is present in the genome as the method is not sensitive enough to detect only one or a few copies of a gene.

Xenopus squashes:-

Batch 2 (neutral pH) 3H-poly-U was used. This was found in Xenopus cells to bind less than batch 1 (low pH)poly-U. Unfortunately there were very few silver grains in the autoradiograms. These are not illustrated

here. Both wild-type and O-nu preparations have sparsely scattered grains over nucleus and cytoplasm, including myofibrils. Very much higher labelling must be obtained before they can be usefully interpreted. As the exposure time is already 10 weeks improvements must be made in the technique, e.g. by using batch 1 (low pH) 3H-poly-U, or higher specific activity poly-U.

5.3. DISCUSSION

The work discussed in the introduction leads to the expectation that in situ hybridization of poly-U will show the cellular location of RNA molecules containing fairly long (possibly 150-200 bases) stretches of poly-A or adenine-rich sequence. In the absence of knowledge of the concentrations of poly-A in the fixed specimens it is not possible to say how adenine-rich or how long the detected sequences are. The poly-A almost certainly represents parts of messenger RNA molecules when detected in the cytoplasm, since free poly-A is not found in cells and only m-RNA and HnRNA contain poly-A. In the nucleus poly-A may represent parts of mRNA or its precursors may also represent parts of HnRNA molecules which are not destined to become cytoplasmic mRNA. The technique has been shown to be capable of detecting poly-A

sequences in Xenopus tissue culture cells and a rat myogenic cell line. Probably for technical reasons there was only a slight amount of poly-A detected in squashes of Xenopus embryos. Ken Jones in this laboratory has also detected poly-A in mouse cells using this technique (in preparation). The possibility of this result being an artefact is reduced by the finding that poly-U which has been RNAsed does not produce labelling of the cells. Also 3H-poly-C does not label cells under these conditions (Ken Jones, pers.comm). Hybrid molecules formed in situ can be extracted and biochemically characterized (John et al 1969).

In both Xenopus cell line and mononuclear rat myoblasts examples were found of preferential labelling of nucleoli, consistent with the findings of Jones (in preparation) using mouse L cells. Nuclear poly-A sequences are found in the HnRNA and mRNA species (see introduction). These findings then imply that HnRNA or mRNA or both associate with the nucleolus. There is some evidence (in Results 5.3) that in rat tissue culture myoblasts this association is only temporary and is confined to the prefused cells. This may reflect an important process in the development of myogenic cells and is evidence in favour of the

possible role of the nucleolus in the processing of mRNA, as is discussed in Chapter 3.

The experiments with Xenopus anucleolar material would have provided relevant information had they worked, and are worth repeating.

In muscle cells, poly-A sequences and hence presumably mRNA are detected in the cytoplasm both before fusion and after the synthesis and assembly of myofibrils. Taken with the findings of other workers (Chapter 3.1) that in similar cultured muscle cells there is a pause in RNA synthesis after fusion, it seems that the RNA synthesized before fusion is stable. As there is no obvious reduction in labelling of nuclei before and after fusion it may be that some mRNAs or precursors or other nuclear RNAs bearing poly-A are stable in the nucleus. Proof of the stability of these RNAs rests on the demonstration that this cell line also ceases RNA synthesis after fusion, or on the results being repeatable on cultured muscle cells in which this has been demonstrated.

Examples are given in the results of myofibrils being preferentially labelled. Thus it seems likely

that myofibrils contain mRNA. This supports the conclusion in Chapter 3 (3H-uridine labelling) that translation of myofibrillar protein mRNAs occurs on the myofibrils. This experiment could now be performed on sections of Xenopus embryo somites in which the myofibrils are clearly demarcated and the labelling of non-myofibrillar sarcoplasm can be directly determined.

The results certainly justify further work, especially repeating the experiments on anucleolar Xenopus cells using low pH poly-U. Although the exposure times are presently too long to allow repetitions of the experiments at the electron microscope level, increasing the activity of the poly-U would certainly pay dividends as it would allow the questions concerning nucleoli to be answered.

5.4. SUMMARY

(1) The technique of in situ hybridization of tritiated polyuridylic acid (3H-poly-U) has been successful in cytologically detecting polyadenylic acid (poly-A) sequences, thought to be attached to mRNA molecules, in a variety of cell types.

(2) The labelling of chromosomes by this technique is much reduced compared with interphase nuclei. However, the result does not distinguish whether or not poly-A is coded in the genome.

(3) Use of this technique has shown that nucleoli in a rat myogenic cell line and in a Xenopus cell line contain poly-A which is consistent with the thesis that one of the nucleolar functions is the processing of mRNA.

(4) Use of this technique has shown that myofibrils contain poly-A. Evidence was presented in Chapter 3 for the existence of newly synthesized, non-ribosomal RNA in myofibrils, as well as the transport of newly synthesized ribosomes to myofibrils. These two findings indicate that mRNA very probably associates with myofibrils. This is probably important in the translation of mRNAs of myofibrillar proteins (see Chapter 3).

CHAPTER SIX

CYTOLOGICAL LOCALIZATION OF RNA POLYMERASE IN EMBYRONIC XENOPUS MUSCLE TISSUE AND A XENOPUS CELL LINE.

6.1. INTRODUCTION

DNA dependent RNA polymerase, otherwise known as nucleotidyl transferase (E.C.2.7.7.6), hereafter called RNA polymerase is the enzyme responsible for the transcription of RNA from the DNA template. It has the requirement for the four substrates ATP, UTP, GTP and CTP, the template DNA and some metal ions referred to below. It is inhibited by actinomycin D (AMD) The template specificities of different isozymic forms of RNA polymerase have been discussed in Chapter 3.1. To summarize:

RNA polymerase I is predominantly nucleolar in location, is more active with ribosomal DNA than bulk

DNA, produces predominantly ribosomal DNA and is activated by magnesium ions.

RNA polymerase II is present in the nucleus but not in the nucleolus. It is more active with bulk DNA than with ribosomal DNA, produces RNA which is DNA-like in its base composition (not rRNA-like) and is activated by manganese and ammonium ions.

These differences are probably due to differences in one of the subunits of the polymeric enzyme. The two types of RNA polymerase have been defined biochemically by extracting them from separated nuclei and nucleoli and identifying them by their products and template specificities, and by different migrations on DEAE Sephadex chromatography. They have also been defined by switching on one or the other form in isolated nuclei by manipulating the salt concentrations and then locating the activity autoradiographically and characterized the product biochemically by extracting it from the nuclear preparation (references in Chapter 3.1). It has been thought possible that the multiple forms of RNA polymerase could serve as a control mechanism in development. Batteries of genes with common promoters could be selectively transcribed by controlling

which form of RNA polymerase was present. Roeder et al (1970) tested this possibility using Xenopus embryos. These embryos do not synthesize ribosomal RNA until gastrulation when the rDNA begins to be transcribed. Embryos homozygous for the anucleolar mutation (Chapter 1) never synthesize rRNA because the ribosomal cistrons are deleted. RNA polymerase was extracted from both types of embryos, before and after gastrulation in wild type embryos, and characterized the enzyme on DEAE Sephadex, by template affinity and by the sensitivity of RNA polymerase II to the drug α aminitin (Lindel 1970). Disappointingly for Roeder and his colleagues both types of polymerase were found in the mutant and were found in the same relative and absolute amounts in pre- and post-gastrula wild type embryos this disproving the theory on developmental control, at least as far as ribosomal genes is concerned.

Nevertheless it was considered interesting to try to find the cellular location of RNA polymerase I (the rRNA synthesizer) in anucleolar Xenopus. Rather than try to isolate the fragile embryonic nuclei for autoradiography, as had been done with rat liver nuclei by previous workers it was decided to attempt to develop an assay for RNA polymerase for use on

whole tissues or sections. This had been achieved in frozen plant sections (Fisher 1968) but only RNA polymerase I appeared to be active regardless of which metal ions were present. While the work was in progress Moore (1971) developed a successful assay using liquid nitrogen and methanol fixation of spermatogenic cells.

The experiments described are not a complete study in that not all of the controls have been performed and optimal assay conditions have not yet been achieved. However some of these preliminary results are promising and interesting and so are reported here. An outline of the development of the technique so far is now presented.

Initially a cultured cell line was used and the cells were fixed in glycerol. This was because the enzyme is stable in glycerol, in fact extracted enzyme is routinely stored in 50% glycerol. The cells must be fixed so that the cell walls become permeable to the nucleoside triphosphates in the assay mixture. It was hoped that glycerol fixation would achieve this. The method worked in that nuclei including nucleoli incorporated 3H-ATP in the reaction, although it was rather awkward as the

glycerol fixation detached the cells from the culture dish and they had to be centrifuged and suspensions spread onto slides for autoradiography. In this first experiment no controls were performed and no magnesium or ammonium ions were present in the assay mixture. The technique was attempted using normal and anucleolar Xenopus embryo pieces. This time AMD was used as a control and RNA polymerases I and II were assayed separately by including Mg or Mn + NH_4 ions respectively in the assay mixture. Some of the embryo pieces were disaggregated and spread onto slides for whole-nucleus preparations and others were embedded in araldite for sectioning and possibly for EM autoradiography if the labelling was high enough. The disaggregated embryo spreads were of a very poor quality, unlike the spreads of cultured cells. The araldite sections were a little better, enabling nuclei and myofibrils to be visualized, but details inside the nuclei were poor. The glycerol fixation was therefore inadequate on cytological grounds for tissue pieces. Also there was incorporation in the AMD control. For these reasons and for the low level of labelling in LMARGs, EM autoradiography was not considered worthwhile.

Progress could still be made by performing further control experiments such as RNAse and AMD sensitivity

of the incorporated label and requirement for all four nucleoside triphosphates using tissue culture cells, provided that a new method of fixation could be found which did not detach the cells from the dish. This is because by this time experiments were so large, having a larger number of variables, that the use of precipitable and spreadable numbers of cells for each variation would require vast amounts of radioisotope, so the cells would have to stay in place in order for the assay to be performed with microlitre amounts of assay mixture under coverslips. To achieve this, glycerol fixation was replaced with lyophilization. Unfortunately this method not only produced a very weak autoradiographic signal with tissue culture cells but when applied to pieces of embryos there was very poor morphology with EM. The project was shelved at this stage. Some of the results gleaned from these preliminary experiments are presented.

6.2. MATERIALS AND METHODS

The Xenopus cell line and its growth conditions were the same as is described in Chapter 5.2.

Xenopus embryos (somite and head regions) of wild type

and anucleolar phenotypes, were obtained as in Chapter 3.2 and sorted as in Chapter 5.2. Hatching stage (Stage 20 Rugh 1948) embryos were used.

Glycerol fixation:-

'Freezing buffer' (0.01M Tris-HCl; 0.1M KCl; 0.1mM EDTA (ethylene diamine tetra-acetic acid); 0.325µl/ml mercaptoethanol; 50%v/v glycerol, pH7.9 before glycerol addition) was poured onto the dishes of cells previously washed in Barth's saline or into small glass tubes containing the Barths rinsed embryo pieces and stood for 40 minutes at 0°. The cells were then rinsed in 5% glycerol in 0.1M Tris-HCl pH7.55 and the embryo pieces were washed in 'washing buffer' (freezing buffer in which the glycerol is replaced with an equal volume of water).

Enzyme reaction:- (modified from Bishop & Robertson 1969). The assay mixture for the Xenopus cells was 0.1M Tris; 0.1M KCl; 2µM spermidine (Koch-Light); 0.5µl/ml mercaptoethanol; 0.7mM K_2HPO_4; 5mM $MnCl_2$; 2.5mM each of GTP, UTP and CTP (Sigma); 1.25mM ATP (Sigma); 200µl/ml 3H-ATP (Radiochemical Centre, Amersham, 500µCi/ml, 20.7Ci/mM); 10% glycerol,pH7.6.

The assay mixture for the embryo pieces was 0.1M

Tris-HCl pH7.5; 0.16mM spermidine; 0.8mM CTP, 0.8mM UTP (Sigma);and 50µl/ml each of 3H-ATP (500µCi/ml, 20.7Ci/mM) and 3H-GTP (500µCi/ml, 9.9Ci/mM, Radiochemical Centre, Amersham). In addition the embryo assay mixture contained either 5mM $MnCl_2$ + 0.4M ammonium sulphate, or 5mM $MgCl_2$. The mixture containing manganese and ammonium was also ± 1µg/ml AMD (Dactinomycin, Cosmogen Lysovac, Merck, Sharpe & Dohme). The radioisotopes, being despatched in a 50% ethanol solution which is inhibitory to RNA polymerase, were lyophilized after dispensation.

The Xenopus cells, still in the dishes, were incubated in approximately 0.1 ml portions of the assay mixture under glass coverslips 1cm square. Pieces of moist tissue were placed in the dishes, the lids put on, and the dishes floated in a 29° water bath for 40 minutes. The embryo pieces were incubated with the different types of assay mixture in small glass tubes, 0.25ml of assay mixture for the trunks and heads of two embryos, at 37° for 40 minutes. The pieces of embryo remained more or less intact but by this time the Xenopus cells had detached from the dishes so that they were pooled and pipetted into 10 ml centrifuge tubes and subsequently treated by pipetting the solution onto them and spinning down the cells

at speed 2 on an MSE bench centrifuge. After the enzyme reaction Xenopus cells were washed in 5% glycerol in 0.1M Tris-HCl pH7.55 and embryo pieces in washing buffer at 0°. All of the preparations were then washed in water and 5% trichloroacetic acid for 10 minutes, at 0°.

Autoradiography:-

Xenopus cells were fixed in 70% ethanol overnight at 4° and then in 3 volumes of ethanol + 1 volume of glacial acetic acid for 15 minutes at 0°, well suspended and pipetted onto gelatinized slides (Chapter 3.2). Light microscope autoradiographic coating and processing was the same as is described in chapter 3.2. Exposure was 1-4 weeks and the autoradiograms were stained with methyl-green pyronin. Photomicrography was done on a Zeiss Ultraphot microscope.

Embryo pieces, after TCA washing were fixed in glutaraldehyde and embedded in araldite as described in Chapter 3.2. Thick (2μ) sections were cut and LM autoradiograms were prepared (exposure times 1-4 weeks), also as described in Chapter 3.2. The autoradiograms were stained in 0.2% aqueous toluidine blue (Gurr) and photomicrography was done on a Zeiss Ultraphot microscope.

6.3. RESULTS

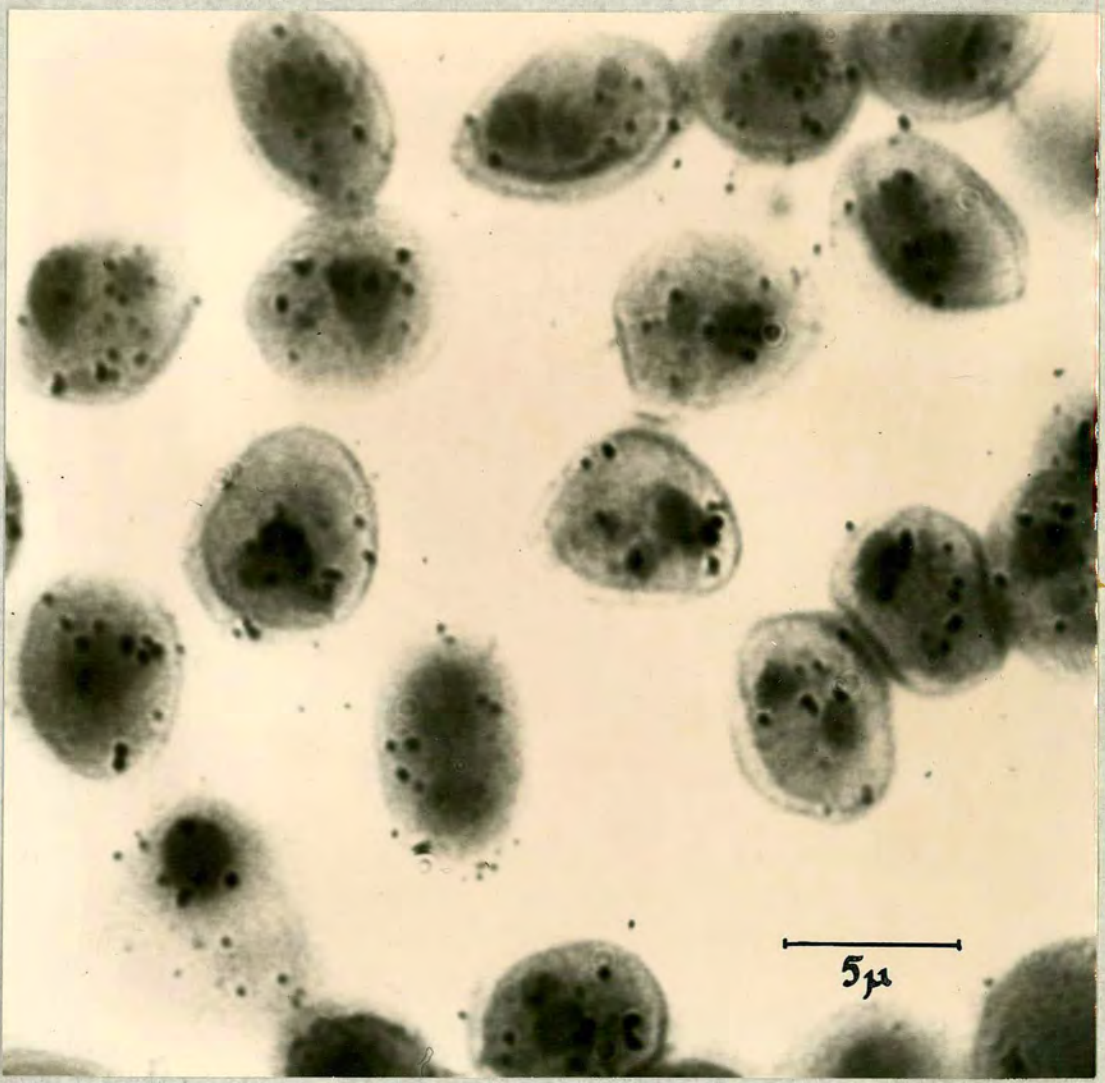


PLATE (1) Xenopus tissue culture cells. Incubation mixture includes manganese. 4 weeks exposure. Experimental details in text. Nuclei and some nucleoli are labelled.

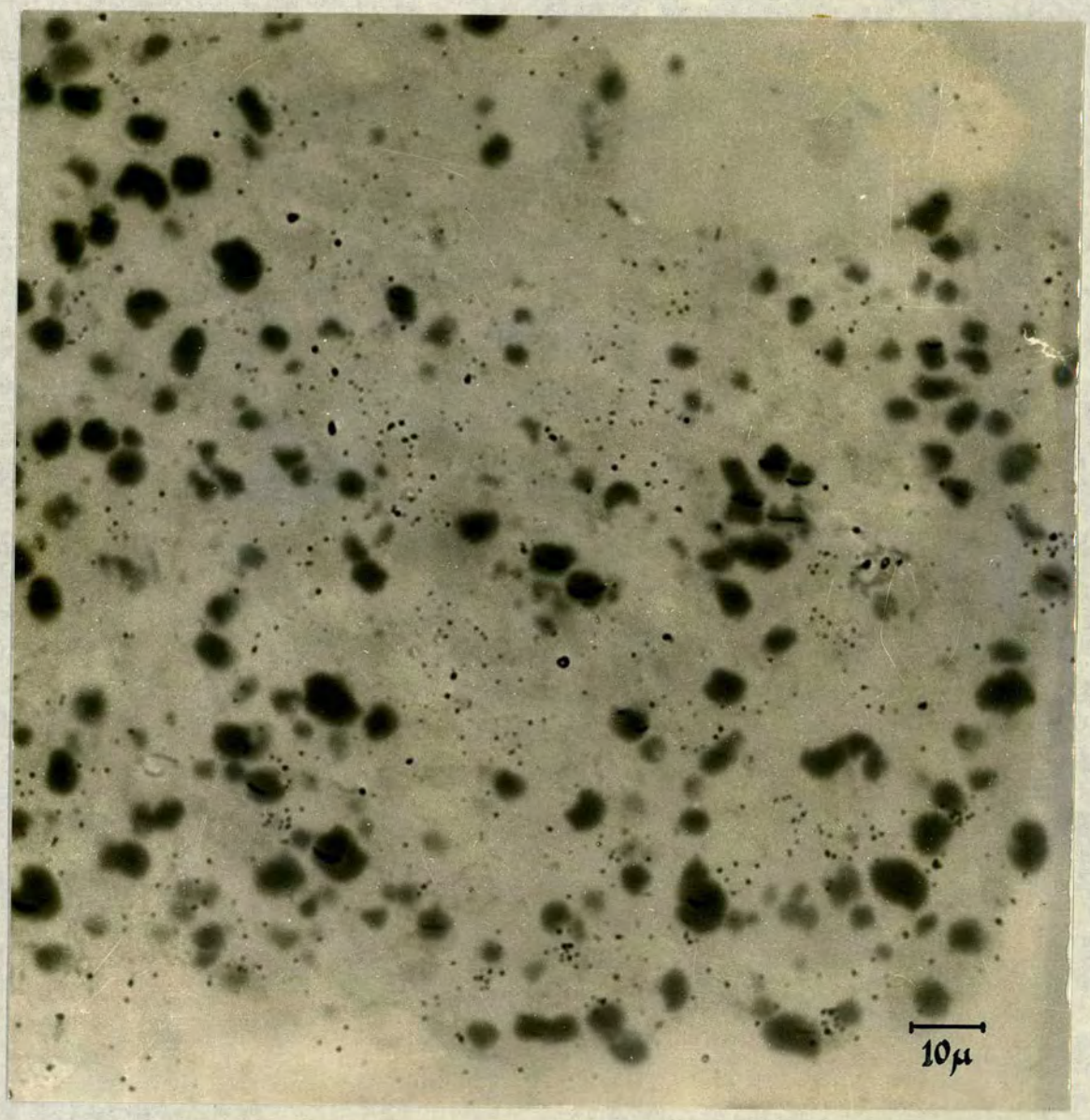


PLATE (2). Wild-type Xenopus embryo. Incubation mixture includes manganese and AMD. 4 weeks exposure. See text for experimental details. Silver grains are present in clusters, possibly over nuclei.

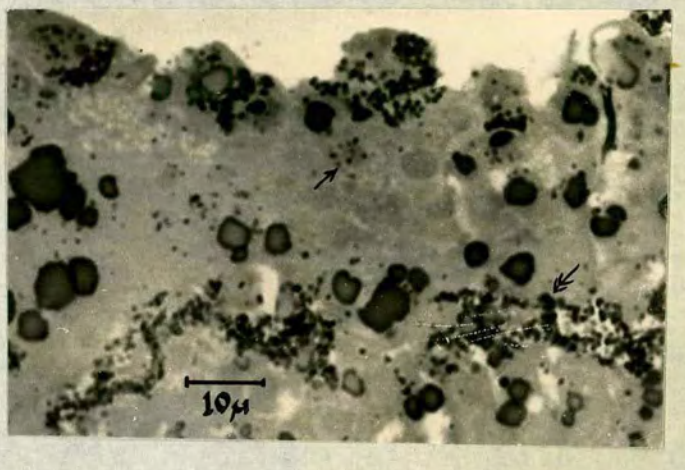


PLATE (3). O-nu Xenopus embryo. Incubation mixture includes manganese. 4 weeks exposure. See text for experimental details. Arrows indicate silver grains (⟶) and pigment granules (⟶⟶).

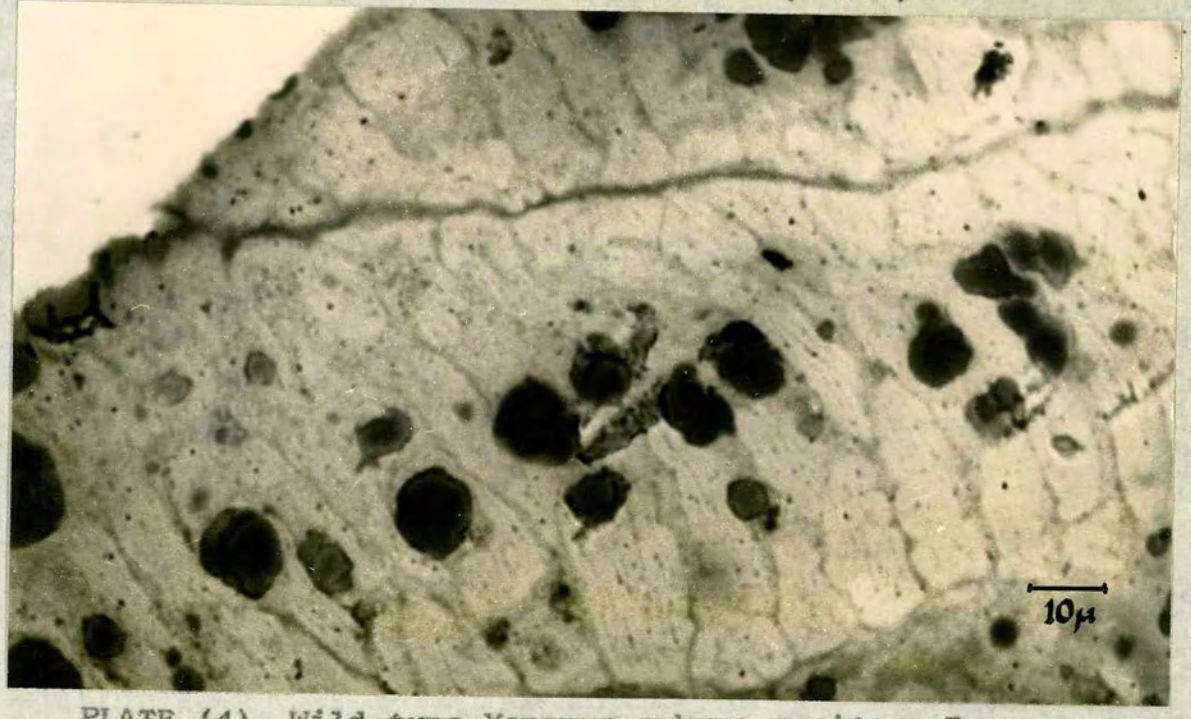


PLATE (4). Wild-type Xenopus embryo somites. Incubation mixture includes manganese. Other experimental details in text. Interfibrillar cytoplasm is labelled.

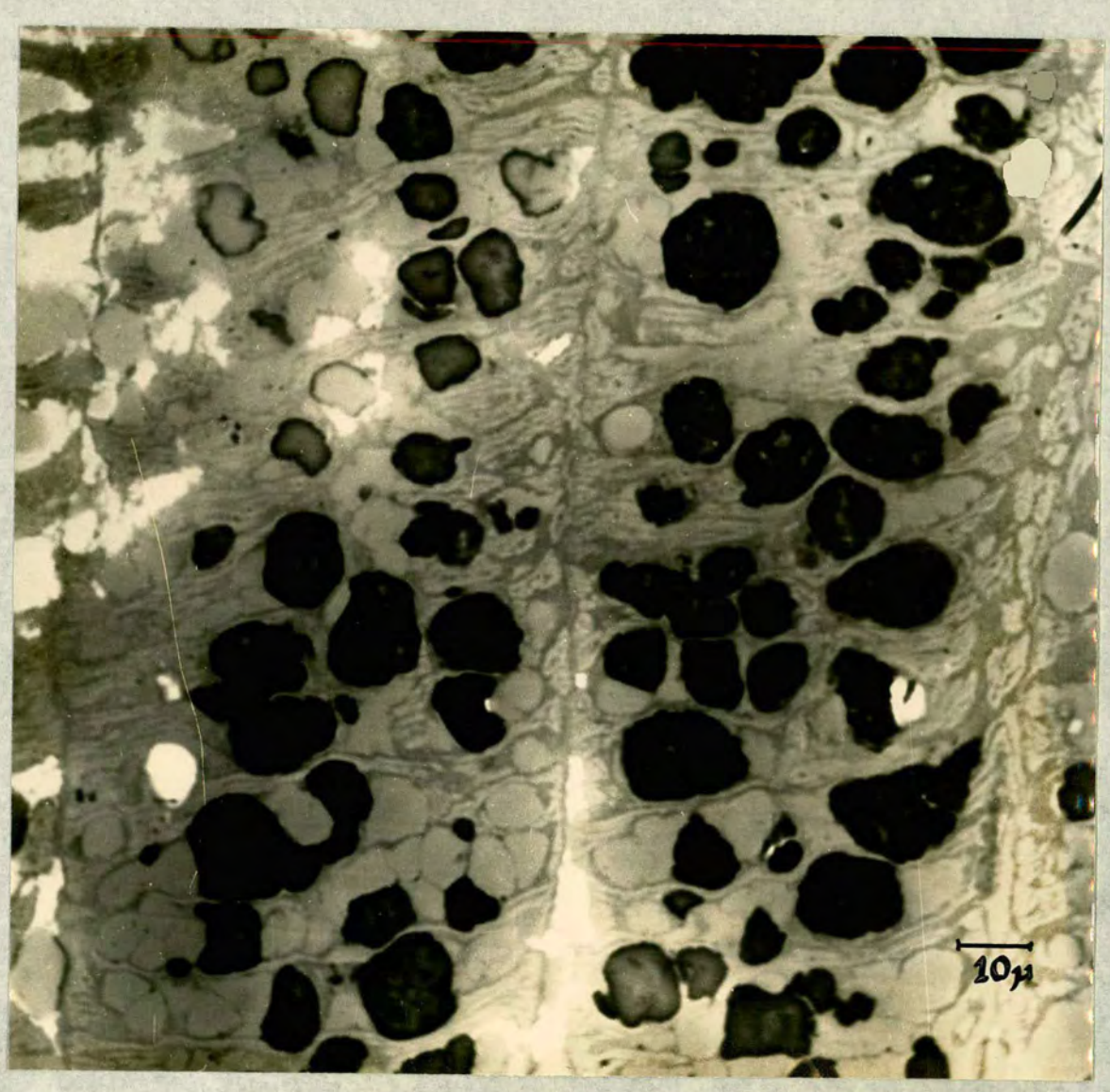


PLATE (5). O-nu Xenopus embryo somites. Incubation mixture includes magnesium. 4 weeks exposure. Other experimental details in text. No silver grains are present.

XENOPUS CELLS:

Plate (1) shows Xenopus cells after being fixed in glycerol and assayed with 3H-ATP plus other enzyme requirements including manganese but no magnesium and ammonium ions. Silver grains are over nuclei and nucleoli with very few extranuclear grains. Some nucleoli are more heavily labelled than others (arrowed). It is not known whether this represents nucleoli lying at different levels below the emulsion, different stages in the cell cycle or some other cause. This result represents a good basis for doing control experiments and trying the technique on embryonic tissue.

CONTROLS:

The only control experiment is the AMD test. RNAse, absence of Mg, Mn and NH_4 and absence of UTP were tried but only in lyophilized cells. Lyophilization did not preserve enzyme activity. Embryo pieces were incubated with Mn + NH_4 ions, with and without AMD. Although with a 1 week autoradiographic exposure some tissues in the material without AMD became labelled (see on) and other tissues in the material with AMD did not become labelled, 4 weeks exposure of the latter did reveal labelling, which although the fixation is poor, is apparently over nuclei, (Plate 2).

Thus the results of these experiments must be viewed with great reserve until further control experiments have been performed.

XENOPUS EMBRYOS:

For reasons of expense of isotopes already referred to, only a little material was available. Nuclei were not always easily visible, particularly in somitic tissue and equivalent tissues cannot always be compared. Silver grains are seen, apparently over nuclei, in pigmented epithelium in 0-nu tissue incubated with manganese ions (Plate 3). The only other significant incorporation observed was in wild-type somites, also incubated with manganese ions, (Plate 4). Here silver grains are distinctly located in the cytoplasm, particularly in the interfibrillar regions. Nuclei cannot be discerned in this piece of tissue. As already described, this apparently manganese requiring incorporation does not appear to be AMD sensitive. Elsewhere there is light labelling of nuclei in some head tissues in wild-type embryos incubated with magnesium ions, but no labelling of 0-nu somites incubated with magnesium ions, where nuclei are not seen (Plate 5). The glycerol fixation was inadequate to reveal intranuclear structures.

6.4. DISCUSSION

In Xenopus cells, as expected, the assay gave incorporation specifically into nuclei. That this incorporation is apparently insensitive to AMD in embryos casts doubts on the specificity of this assay. Thus no firm conclusions can be reached. However the exciting observation of cytoplasmic incorporation in wild-type muscle cells in the interfibrillar regions, implying cytoplasmic transcription, at least justifies repeating the experiments with AMD and searching for a better fixative (e.g. the liquid nitrogen and methanol fixative method of Moore 1971) compatible with good morphology and enzyme activity, such that the activity of anucleolar blobs can be ascertained. There are some other justifications. These are the specificities of the labelling patterns observed. Xenopus cells show specifically nuclear labelling. Wild-type somite cytoplasm labels when manganese ions are present but O-nu somites with magnesium ions do not. In an epithelial tissue in O-nu embryos incubated with manganese nuclei are again apparently specifically labelled. Thus although homologous comparisons cannot be made it appears that the activity observed has some kind of specific ion requirement and some kind of cytological localization.

6.5. SUMMARY

A cytological assay for DNA dependent RNA polymerase has been attempted. The results obtained conform to expectations with Xenopus tissue culture cells in that the activity is in the nucleus. In muscle cells some instances were found of cytoplasmic activity, implying that mRNA is transcribed on a cytoplasmic template. The significance of this finding and other relevant findings is discussed in Chapter 7.2. These preliminary results show that a more thorough study would be rewarding, particularly if concentrated on studying the AMD and RNAse sensitivity of the assay and on finding fixation techniques more compatible with both enzyme activity and intranuclear morphology.

CHAPTER SEVEN

APPENDIX

7.1. MYOSIN & DNP OF DYSTROPHIC MUSCLE

Introduction

Materials and Methods

Results

Discussion

INTRODUCTION

In other parts of this thesis some evidence is presented for the existence of non-mitochondrial DNA in muscle cell cytoplasm; Chapter 3 presents some evidence for cytoplasmic transcription, Chapter 4 describes thymidine labelling and Chapter 6 presents some evidence for the existence of DNA dependent RNA polymerase in muscle cell cytoplasm. In this section independent evidence was sought in a different organism. Dr Hew John (unpublished) discovered an RNAse resistant substance with a nucleic acid-like UV absorption spectrum coprecipitating with myosin from dystrophic mouse muscle, and chromatographically separable from the myosin. The substance did not appear when myosin was extracted from normal muscle. This presumptive DNA or DNA-protein complex (DNP) was not from whole nuclei

or whole mitochondria as these were removed early in the extraction procedure by centrifugation. The fact that the presumptive DNP coextracts through three serial precipitations of myosin suggests that it may be functionally attached to the myosin in vivo. An alternative explanation is that DNA is released from nuclei and/or mitochondria during extraction and forms a fairly tight but non-covalent attachment to the myosin. To test these hypotheses liver tissue was extracted with carrier myosin which had been chromatographically purified.

MATERIALS & METHODS

Myosin extraction is the method of Dow & Stracher (1971) modified by Dr H.John (unpublished). The extraction plan is shown on the last page of Materials and Methods. A normal and a dystrophic young mouse from the same litter produced by crossing heterozygotes obtained from C57 and 129RE stock were used. They were killed by stunning and breaking their necks. Livers were removed, the gall bladders removed from the livers, and the livers were stored on ice. The feet were cut off and the skin removed from the body and legs. The pectoral and pelvic limb girdles, including limbs, bone and some vertebral associated

tissue, were removed and stored on ice. The tissues were left on ice for 15 minutes to thoroughly chill, then briefly rinsed in phosphate buffer (0.4M KCl, 0.01M sodium pyrophosphate, 0.005M $MgCl_2$, in 0.05M pH 6.5 phosphate (100:40 v/v ratio of equi-molar KH_2PO_4 and K_2HPO_4) and weighed. The tissues were finely chopped with scissors and extracted with 3 volumes of phosphate buffer for 20 minutes at 4° with stirring. The debris, including whole cells, membranes, nuclei and mitochondria, was centrifuged off at 20,000g for 10 minutes, the lipid pellicle filtered off with gauze and the supernatant volume measured. The supernatants from the liver extracts were divided into two equal volumes and 1mg of chromatographically purified mouse myosin (see below) was added to one of the fractions of dystrophic liver extract and one of the fractions of the normal liver extract, (into 1ml. of dystrophic liver extract and into 2 ml. of normal liver extract). Both liver and muscle extracts were dialyzed (Visking tubing) against 14 volumes of water at 4° for 4 hours, which precipitated crude myosin from muscle extracts. The precipitates were centrifuged off at 20,000g for 10 minutes. All preparations had a visible pellet at this stage. The precipitates were redissolved in $\frac{1}{2}$-$\frac{1}{3}$ of the original extraction volume of phosphate buffer. The solution

from the liver extracts remained cloudy despite thoroughly mixing with a Teflon-glass homogenizer, perhaps because of the presence of glycogen particles.

In order to remove RNA which may be present in the extracts, not less than 0.5mg of RNAse (RNAse A, Sigma) was added to each extract (giving 0.25-0.5mg/ml. RNAse) and dissolved by stirring. These were incubated at 20° for 5 minutes and then at 4° for 18 hours.

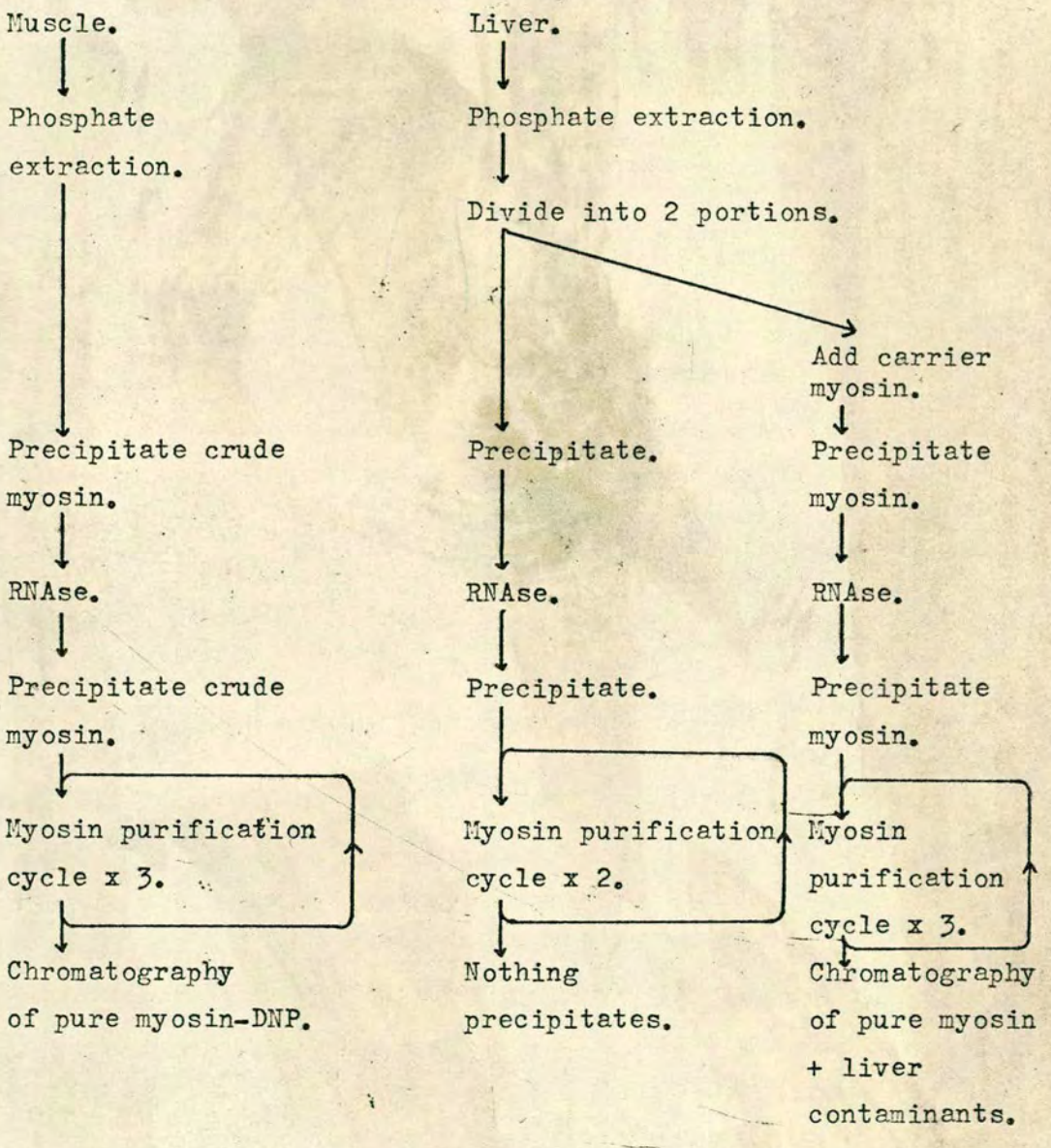
The myosin was reprecipitated by dialyzing against 14 volumes of 0.5mM dithioethreitol for 4 hours at 4°. The myosin was centrifuged off at 20,000g for 10 minutes. All preparations had a precipitate. The myosin was then taken through the following purification cycle three times, mainly to remove actomyosin. The pellet was dissolved in $\frac{1}{4}$-$\frac{1}{2}$ of the original extraction volume of myosin B solution (0.81M KCl, 0.11M KH_2PO_4, 0.1M K_2HPO_4, 1mM EDTA, 0.5mM dithioethreitol, pH6.5). As the liver preparations were still cloudy, all preparations were, at this stage, clarified by centrifuging at 20,000g for 10 minutes, on the first cycle only. These solutions were instantaneously diluted with 3 volumes of water from a blown pipette and centrifuged at 20kg for 10 minutes. Myosin was precipitated from the supernatant by dialyzing against

7 volumes of DTE for 4 hours at 4° and centrifuging at 20,000g for 10 minutes. END OF CYCLE.

On the first cycle there was no visible pellet in both of the liver extracts minus carrier myosin and on the second cycle 1ml. washings from the bottoms of the tubes of liver extract minus carrier had less than 0.040D per cm. at 260 and 280nm. so they were discarded.

After the third cycle pellets were dissolved in a minimum volume of myosin B solution for chromatography, and clarified by centrifuging at 43,500g for 15 minutes. ODs of the samples are recorded in the results. The myosin solutions, at less than 1% concentration (=5.6 OD/cm at 280nm) were loaded onto a 25x1.5cm diameter column of Sephadex equilibrated with 0.5MKCl, 0.0005M dithioethreitol in 0.02M Tris pH 7.3 with HCl, and run at 15ml/hour at 4°. 0.5ml fractions were collected and the ODs measured at 260, 280 and 320nm on a Beckman spectrophotometer. The fractions from the muscle extracts presumed to contain nucleic acid because of the high 260-280nm. absorbancy ratio and equivalent fractions from the liver extracts were pooled and concentrated by dialyzing against equal volumes of glycerol at 4° for 24 hours. The contents of the dialysis tubes were put into fresh

PLAN OF EXTRACTION PROCEDURE. FOR MYOSIN-DNP.



dialysis tubes which were tightly knotted and dialyzed in a large volume of water at 4° for 45 hours to remove glycerol. This achieved a 50-75% reduction in volume.

An attempt was made to estimate the DNA and RNA contents of the samples. RNA was estimated by precipitating total nucleic acids and proteins with 0.5 volumes of 0.2N perchloric acid (PCA) at 0° for 15 minutes, centrifuging at 10,000g for 5 minutes and washing the precipitate 2x15 minutes in 0.2N PCA. 1ml of N-PCA was added to each precipitate and stood for 22 hours at 0°, to hydrolyze RNA, and this was centrifuged at 10,000g for 5 minutes to remove the DNA and protein precipitate. The ODs of the supernatants (RNA) were read at 260, 280 and 320nm. (see results) on a Unicam SP800 spectrophotometer. To estimate DNA, 1ml. of N-PCA was added to each precipitate and these were heated at 80° for 30 minutes to hydrolyze DNA, cooled to 0°, centrifuged at 10,000g for 5 minutes to remove the protein precipitate, and similar OD measurements made on the supernatant (see results). Because of the low yield from single animals, however, there were no visible PCA precipitates so the very low optical densities probably may not be meaningfully compared.

RESULTS.

Figures 1-4.

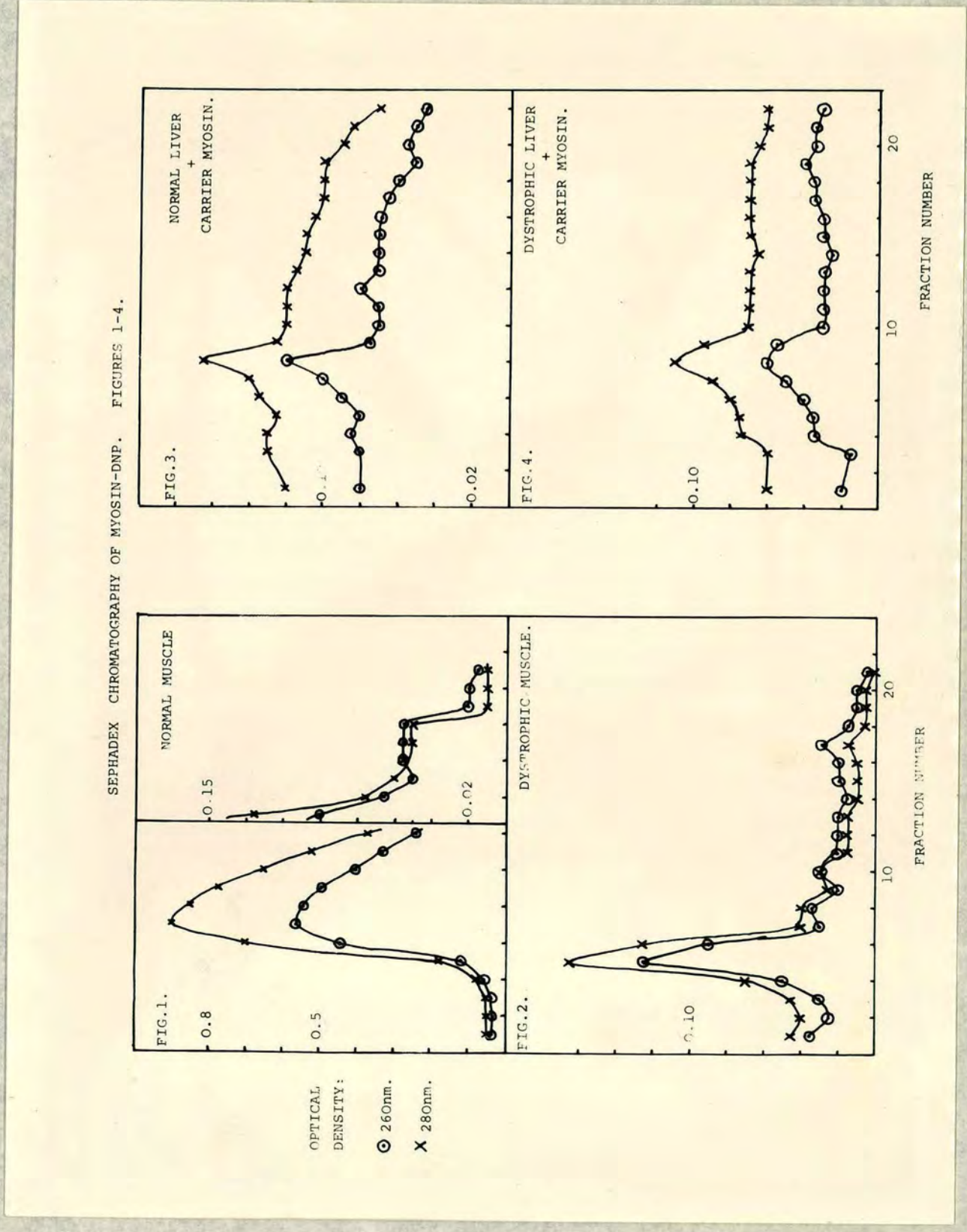


TABLE 1. Yield of Triple Purified Myosin from Various Sources

Optical densities calculated for 1gm wet weight of tissue extracted into 1ml.

Wavelength (nm.)	260	280	320	$\frac{OD280}{OD260}$
Normal muscle	0.640	0.720	0.044	1.1
Dystrophic muscle	0.270	0.377	0.035	1.5
Normal liver (2xpurified)	0.062	0.046	<0.001	0.75
Normal liver + myosin	0.262	0.262	0.038	1.0
Dystrophic liver (2xpurified)	<0.001	<0.001	<0.001	-
Dystrophic liver + myosin	0.371	0.400	0.071	1.1

TABLE 2 DNA & RNA Estimates of Presumptive DNP.

Column	1	2	3	4	5
	$\frac{OD280}{OD260}$	OD260 RNA	OD260 DNA	RNA/g	DNA/g
Dystrophic muscle	0.50	<0.001	0.016	<0.091	0.146
Normal muscle	0.91	0.014	0.025	0.006	0.010

Column 1 is taken from fraction 17 of the chromatographs (Figs 1&2). For columns 2&3, 1ml. of the 11.7ml. of the concentrate of fractions 15-18 inclusive (Fig.2) from dystrophic muscle (corresponding to 0.11g out of the original 1.3g. wet weight of muscle) was used and 0.75ml. out of 1.5ml. concentrate of fractions 15-18 inclusive (Fig.1) from normal muscle (corresponding to 2.5g. out of 5g.) was used in DNA-RNA estimations as described in materials and methods. Columns 4&5 give the same figures as columns 2&3 on a per gram wet weight starting material basis.

Table 1 shows that no detectable myosin was extracted from the liver but that there was a good yield from liver tissues from which myosin had been added. The OD280/OD260 ratio shows that the extracts are not, however, pure protein; nucleic acid seems to be present.

When chromatographed on Sephadex G200 the extracted, triple purified myosin appears (Figs 1-4) as a sharp peak with a high 280:260nm absorbancy ratio indicative of protein, peaking at fractions 5-8. This peak is in all cases followed by a considerable shoulder spreading up to fraction 20. There is a marked difference between liver and muscle tissue with respect to this shoulder region. In the liver tissue (with carrier myosin) in Figures 3 and 4, whether normal or dystrophic, the OD at 280nm remains at about twice the level of the OD at 260nm, as in the myosin peak, indicating the presence of protein with little, if any nucleic acid. The nature of this protein is not known. However, with normal muscle (Fig.1 and Table 2) the 280/260nm. absorbancy ratio in the shoulder approaches unity, indicating an excess of nucleic acid over protein, and in the dystrophic muscle the 280/260nm. ratio falls to 0.5 in many places (Fig.2 and Table 2) indicating the presence of highly purified nucleic acid.

It appears then that this RNAse resistant presumptive nucleic acid only coextracts with myosin from muscle tissue and not from liver tissue, and when chromatographed the nucleic acid separates more cleanly from protein from dystrophic muscle than from normal muscle. However it may be that nucleic acid is present in the liver extracts and that the protein in the shoulder region of the chromatogram effluent is masking this nucleic acid. As nucleic acids have about 20 times the OD of the same mass of protein at 280nm there must be very much less, if any DNA in the liver extracts than in the muscle extracts. PCA hydrolysis estimation of nucleic acids was unsatisfactory as the yield was too small to produce a precipitate. The estimation, although having unreliably low ODs,does show that the nucleic acid is mainly DNA (Table 2).

DISCUSSION.

A substance with a high 280:260nm absorbancy ratio, resistant to RNAse and presumed therefore to be DNA or DNP co-extracts with myosin from normal and dystrophic muscle and is separable from myosin by gel chromatography. The presumtive DNA from dystrophic myosin separates as probably pure, protein-free DNA whereas that from normal muscle is not completely separated from protein. That this finding is probably

not an artefact produced by the binding of DNA from ruptured mitochondria and/or nuclei is shown by the high 280-260nm OD ratio of equivalent regions on the chromatograms of liver extracts containing carrier myosin. However the presence of relatively large amounts of protein in these regions may be masking the presence of some artefactual DNA. The artefactual DNA in liver, if present, must be very much lower in quantity than that observed in muscle. PCA hydrolysis estimation of nucleic acids on this presumptive DNA from muscle tissues was unreliable because of the small yield but did show a preponderance of DNA.

This work justifies further experiments, most importantly the repeat of extracting liver with carrier myosin to obtain a high enough yield to estimate DNA, RNA and protein. The nuclease sensitivity of the purified presumptive DNA from muscle tissue must also be checked. Yields can obviously be improved now that the existence of DNA is more certain, by extracting DNA directly from the cytoplasm of muscle cells: presumably the extraction conditions for myosin are not optimal for DNA extraction. As the presence of this DNA has been shown in normal muscle this tissue can now be used to obtain greater amounts of material. Electron microscopy of myosin-DNP and purified DNP

(i.e. before and after chromatography) would be a worthwhile project.

The significance of cytoplasmic DNA in muscle tissue is discussed in Chapter 7.2.

APPENDIX 7.2

GENERAL DISCUSSION ON CYTOPLASMIC TRANSCRIPTION

One aspect of the work in this thesis which deserves special discussion is the evidence presented for cytoplasmic transcription. Evidence for this comes mainly from Chapter 3 concerning the labelling kinetics of tritiated uridine in myofibrils; from Chapter 4 concerning the labelling of muscle cytoplasm with tritiated thymidine; from Chapter 6 concerning a cytological DNA-dependent RNA-polymerase assay and from Chapter 7.1 concerning the coextraction of an RNAse resistant nucleic acid with myosin from mouse tissues. Each case is discussed here in its new context, briefly recapping the quality of the evidence.

KINETICS OF INCORPORATION OF TRITIATED URIDINE INTO MYOFIBRILS

In Chapter 3 it was shown that the myofibrils of both wild-type (WT) and anucleolar (0-nu) Xenopus somites incorporate tritiated uridine (3HU). The myofibrils of 0-nu Xenopus incorporate 3HU linearly with time for at least 60 minutes. WT myofibrils, however, incorporate 3HU at about the same linear rate for only 30 minutes and then the rate of uptake increases such

that they have about twice the specific activity of O-nu myofibrils at 60 minutes. The direct interpretation is that newly synthesized ribosomes begin to attach to WT myofibrils between 30 and 60 minutes after they are synthesized in the nucleolus, and that the labelling of myofibrils in WT up to 30 minutes, and all the time in O-nu represents non-ribosomal RNA.

If the non-ribosomal RNA is nuclear in origin a lag in labelling of myofibrils would be expected, as is apparently the case for rRNA, representing the time required to synthesize, process and transport the RNA from the nucleus to the myofibrils. In the organism in which RNA metabolism has been probably most intensively studied; the HeLa cell, it takes at least 15 minutes, and probably nearer to 45 minutes for newly synthesized RNA to reach the polysome pool, (e.g. Penman et al 1968). In rat tissue culture, myoblasts Yaffe & Fuchs (1967) found that in pulses of 3HU of up to 40 minutes almost all of the grains in autoradiographs were associated with the nucleus and that substantial numbers of grains only appeared in the cytoplasm in pulses of longer than 40 minutes. After this, cytoplasmic incorporation was linear with time. No such lag in incorporation of 3HU into the cytoplasm is observed in these experiments. O-nu myofibrils are

significantly labelled after a 30 minute pulse and WT myofibrils after a 10 minute pulse.

It is still possible that the myofibril-associated non-ribosomal RNA is nuclear in origin but that it exits from the nucleus in less than 10 minutes after synthesis begins. Evidence against this comes from observations on the labelling kinetics of nuclei. In muscle nuclei a significant decrease in specific activity during a chase after a pulse of 3HU is not observed in pulses of up to 60 minutes and chases of up to 6 hours, both in WT and in 0-nu. In WT the nucleolus is probably acting as a reservoir of labelled RNA during the chase, maintaining the specific activity of the nucleoplasm despite losses of rRNA to the cytoplasm, but in 0-nu no such reservoir exists. Therefore in these short pulse times no detectable non-ribosomal RNA travels out of muscle nuclei, during which time the myofibrils become significantly labelled. The nucleus then cannot be the synthesis site for all of the myofibril-associated non-ribosomal RNA, particularly as nuclei occupy a much smaller volume than myofibrils such that a very large fall in the specific activity of nuclei during a chase would be required to account for even a small uptake by myofibrils.

The results, then, taken at face value, indicate that myofibril-associated non-ribosomal RNA in Xenopus somites cannot be satisfactorily accounted for in its origin in the nucleus and so is probably either synthesized in the myofibrils or in the surrounding cytoplasm.

There are reservations to this conclusion, concerning the specificity of labelling and the efficiency of chasing. It has not been conclusively demonstrated that the uridine labelling represents RNA synthesis. For instance in these experiments RNAse sensitivity could not be demonstrated. However, other evidence is presented in Chapter 3 which shows that it is highly likely that the labelling does represent newly synthesized RNA. The other reservation concerns efficiency of chasing. If there is a substantial concentration of free 3HU remaining in the tissues during a chase experiment, say a comparable concentration to that used in the pulse, then the conclusion concerning the stability of nuclear non-ribosomal RNA is invalidated. Again evidence is presented in Chapter 3 for the efficiency of chasing being very good, notably the fact that non-muscle nuclei in WT chase to a very low specific activity after a pulse.

INCORPORATION OF TRITIATED THYMIDINE INTO MYOFIBRILS

In Chapter 4 it is shown that tritiated thymidine is incorporated into myofibrils. The labelling of myofibrils is quite distinct in these experiments from labelling of mitochondria, which are known to replicate DNA in the cytoplasm. In other cell types, no cytoplasmic labelling is observed in light-microscope autoradiograms. The incorporated product is not sensitive to TCA treatment and so is of high molecular weight and not thymidine non-covalently bound to other cell components. Cytoplasmic labelling due to nuclear damage cannot be ruled out but evidence against this is presently in Chapter 4. The direct conclusion is that newly synthesized DNA is associating with myofibrils. As with uridine labelling, thymidine uptake into myofibrils begins at the shortest pulse time used (10 minutes) and nuclei do not show any appreciable fall in specific activity in chases up to 6 hours, so the myofibril-associated DNA appears to be cytoplasmic in origin.

Again there is no conclusive evidence that the thymidine labelling represents DNA synthesis, as the labelling is not sensitive to DNAse. However, it is considered likely to represent DNA synthesis, as is discussed in

Chapter 4, mainly on the grounds that thymidine is a direct precursor of DNA and that the labelling is insensitive to the same actinomycin D treatment which abolishes RNA synthesis.

CYTOLOGICAL RNA POLYMERASE ASSAY

DNA-dependent RNA polymerase (RNA polymerase) is the enzyme which catalyzes the transcription of RNA from the DNA template. In Chapter 6 it was described how a cytological assay was attempted on whole tissues of Xenopus embryos, in order to determine the distributions of the various forms of the enzyme relative to nucleoli and blobs. The attempt was only partially successful in that fixation conditions compatible with enzyme activity and with good morphology have not yet been found. Neither have incubation conditions completely specific for the enzyme been found. However, in WT Xenopus somites, interfibrillar regions of the cytoplasm did become labelled, showing that RNA polymerase is possibly present there. The incorporation in nuclei does not appear to be sensitive to the presence of actinomycin D, an inhibitor of the enzyme, indicating that the assay is not necessarily specific for the enzyme in nuclei. However, 0-nu muscle cytoplasm does not become labelled when the metal ion content of

the assay mixture is changed, indicating that the sarcoplasmic incorporation is specific to either a particular genotype or a particular ionic environment; the labelling is not entirely non-specific. It is possible then that muscle cytoplasm contains DNA-dependent RNA-polymerase.

The evidence from thymidine labelling (see above) suggests that DNA is the natural primer for this enzyme. As primer is not included in the assay mixture it is possible that RNA is priming the enzyme, giving the observed activity as it is known that the enzyme has 10% of the activity with RNA as primer instead of DNA (see refs. in Chapter 6). Further work would be needed to resolve this question. If RNA is priming the enzyme in the in-situ assay, this would explain the insensitivity of the reaction to AMD, as this inhibits the reaction by binding to DNA. However, Bell et al (1972) shows that newly synthesized RNA associates with the DNA particles isolated from muscle cell cytoplasm.

Since the rest of this thesis was written Bell & Brown (1972) have isolated this enzyme from embryonic chick muscle cell cytoplasm, supporting my autoradiographic observations in Xenopus somites.

MYOSIN-DNP OF MOUSE MUSCLE

In Chapter 7.1 it is shown that a nucleic acid co-extracts with myosin from mouse muscle tissue. The nucleic acid is resistant to exhaustive RNAse treatment of the crude extract. When eventually separated from the myosin by chromatography the presumptive DNA fractions from dystrophic mouse myosin are freer from protein than those separating from normal mice. When carrier myosin is added to a similar extract from liver and the myosin extraction procedure performed, the re-extracted myosin is not contaminated with nucleic acid, showing that myosin is probably not binding DNA released from broken nuclei or mitochondria during the extraction. These findings imply a functional association of myosin and DNA possibly coupled with other proteins in dystrophic mouse muscle <u>in vivo</u>. Confirmation of the findings awaits rigorous demonstration that the substance coextracting with myosin is DNA.

Thus several independent lines of investigation each support the hypothesis that in muscle tissue non-ribosomal RNA is transcribed in the cytoplasm on a DNA template. As is often the case in biological studies, the individual findings are not rigorously

confirmed, but this is usually because of lack of data rather than direct evidence against them.

The hypothesis does find positive support in the work of other authors, principally the discovery of rapid incorporation of tritiated thymidine into a 7S nucleic acid within the 16S nucleoprotein particle in the cytoplasm of embryonic chick muscle by Bell (1969). As discussed in Chapter 4, this appears to represent the presence of cytoplasmic DNA. The work of Bell has been unfavourably received by the scientific community in general but this is mainly because of the authoritative denunciation of all other theories of eukaryotic protein synthesis. There does not appear to be any direct evidence against the existence of this DNA.

Amplification of specific genes, although not cytoplasmic, is well documented for amphibian oocytes (e.g. Gall 1968, Brown & Dawid 1968). Pelc (1968) reviews numerous cases of what he considers to be gene amplification. He defines 'metabolic DNA' as DNAsynthesized in excess of that required for cell division or permanent polyploidy, and includes cases where this excess DNA appears to travel to the cytoplasm. Another example cited by Pelc is that of organ (muscle) specific differential synthesis of low molecular weight DNA in the mouse.

It could be argued that cytoplasmic DNA, if it exists, should have been discovered long ago in routine DNA extractions. If it is present, the reason that it could have been missed is that it may be only a very short molecule compared with chromosomal DNA as it would probably only contain one or a few genes. For example the DNA encoding myosin would, presumably, have a sedimentation coefficient of the same order as myosin-mRNA, (26S). It may also be tissue specific. Most DNA extraction methods only extract high molecular weight DNA.

If muscle cytoplasm does contain DNA, cytoplasmic transcription would also require RNA polymerase to be present. In all of the previous studies on RNA polymerase in eukaryotes, described in Chapter 3.1, isolated nuclei were used for autoradiography or extraction. Thus the presence of cytoplasmic RNA polymerase could have been overlooked. Thus there is no direct evidence in the literature cited against the preliminary findings reported here. Bell & Brown (1972) have since isolated this enzyme from muscle cytoplasm.

If cytoplasmic transcription does occur in muscle tissue it might be expected that it would have been more widely observed. If present it may have been missed because in most cases the RNA product would

only be a minor species, as there are few cases in which messenger RNA synthesis takes place in the absence of a great excess of ribosomal RNA synthesis, as is so in the anucleolar Xenopus embryos. Cytoplasmic transcription and/or gene amplification may be peculiar to certain genes such as the ribosomal genes already mentioned and to certain tissues such as muscle and lens which produce relatively massive amounts of a few proteins, such that a few genes are in great demand. As already mentioned and fully discussed in Chapter 3.1, non-ribosomal RNA metabolism has been very extensively studied in HeLa cells by selectively suppressing rRNA metabolism with low doses of actinomycin D (AMD) by Penman's group. These cells are fairly non-specialized tissue culture cells of tumour origin and no-one has suggested that cytoplasmic transcription occurs in them, but a thorough series of studies has so far failed to prove that polysome associated cytoplasmic messenger RNA originates in the nucleus (Penman et al 1968, Darnell et al 1970). Nuclear RNA has a much higher degradation rate, a higher modal molecular weight and a greater heterogeneity of molecular weight than cytoplasmic messenger RNA. The two types of RNA competitively hybridize with DNA (Soeiro & Darnell 1970) but the overlap is probably in the reiterated parts of the molecules which do not necessarily form specific

hybrids (Darnell et al 1970), showing that they were possibly not transcribed from the same segments of DNA. Thus the hypothesis that mRNA is nuclear in origin is not yet proven even in HeLa cells.

One aspect of the conclusions from the work in this thesis then is that evidence can be found for the existence of non-ribosomal, non-mitochondrial, cytoplasmic DNA in muscle which associates with myofibrils and may be used in the transcription of RNA which also associates with myofibrils. The work of other authors supports this hypothesis and the widely held view of the nuclear origin of messenger RNA is not considered proven.

APPENDIX 7.3

SUMMARY OF DATA FOR CHAPTER 3

Specific activities are calculated by counting silver grains and weighing paper tracings of cell components in autoradiograms as described in Chapter 3.2. The formulae used are:

$$A = \frac{wx10^6}{dxM^2}$$

where g = number of grains

A = area in μm^2

w = weight of paper (g).

d = density of sheet of paper in g/mm^2 (each sheet weighed whole)

M = Magnification factor (linear)

s = specific activity in grains/μm^2

The standard error of each mean specific activity, taking each photograph as a unit observation (regardless of how many pieces of a given cell structure are present in each photograph) was computed thus:

$$V(\hat{s}) = \frac{1}{s(A)^2} \sum_{n=1}^{n} (g_i - sA_i)^2$$

$$S(\hat{s}) = \frac{1}{n(A)^2(n-1)} \sum_{n=1}^{n} (g_i - sA_i)^2$$

$$= \frac{1}{n(A)^2(n-1)} (S_{yy} - 2sS_{xy} + s^2S_{xx})$$

$$S_{yy} = \sum_{n=1}^{n} \frac{(g_i - \bar{g})^2}{n-1}$$

$$S_{xx} = \sum_{n=1}^{n} \frac{(A_i - \bar{A})^2}{n-1}$$

$$S_{xy} = \sum_{n=1}^{n} \frac{(g_i - \bar{g})\ (A_i - \bar{A})}{n-1}$$

$$s = \hat{s} \pm 1.95\sqrt{S(\hat{s})}$$

$\overline{A^2}$ = mean square area

V = variance

S = standard error

s = true specific activity

$\hat{s}$ = the estimate from the sample giving 95% confidence limits

Standard errors of observations are shown in Table 1 in Chapter 3.2. This formula was provided by Mr Shukla of the Statistics Department of Edinburgh University, and the computation was programmed by Miss Cathy Paver of this department.

In the following tables the data for Table 1 in Chapter 3,2 is presented, giving the number of plates analyzed, total numbers of grains counted, total areas in μm^2 measured and specific activities in grains/μm^2 for each cell structure in each experiment and for background. Cell type and experiment code abbreviations are explained in Chapter 3.2. Also presented is a summary of the data and X^2 tests for the sarcomere profiles of Chapter 3.2 where the methods for obtaining these are described.

NUCLEOPLASM

12 weeks exposure. (* is 4 weeks, ** is corrected to 12 weeks).

EXPERIMENT	CELL	PLATES	GRAINS	AREA	SPEC.AC
10+0	U+	3	13	277.2	0.0469
	M+	2	3.5	115.8	0.0302
10+30	U+	3	37	208.3	0.1776
	M+	5	37.5	328.2	0.1143
30+0	UO	2	12	211.9	0.0566
	MO	7	18	499.9	0.0360
30+0	U+	3	19.5	87.6	0.2340
	M+	3	18.5	202.9	0.0912
30+30	UO	1	6.5	9.6	0.6757
	MO	4	40.5	411.4	0.0984
30+30	U+	5	19.5	910.0	0.0214
	M+	6	40.5	364.1	0.1112
60+0	UO	4	50.5	1441.4	0.0350
	MO	6	48.5	618.3	0.0784
60+0	U+	6	141.3	657.5	0.2151
	M+	1	29.3	135.25	0.2166
60+6h	UO	5	77.5	2775.6	0.0279
	MO	2	28.5	175.3	0.1626
60+6h	U+	4	12	386.1	0.0311
	M+	4	103.3	498.4	0.2073
15+0*	MO	4	37	241.5	0.1532
**					0.4500
15+0*	M+	6	170.3	602.2	0.2828
**	M+				0.8400

NON-MYOFIBRILLAR CYTOPLASM

12 weeks exposure (*is 4 weeks, ** is corrected to 12 weeks)

EXPERIMENT	CELL	PLATES	GRAINS	AREA	SPEC.AC.
10+0	U+	3	3	382.0	0.0079
	M+	5	15	257.8	0.0582
10+30	U+	1	6.5	228.0	0.0267
	M+	5	35.5	752.7	0.0472
30+0	UO	2	3	100.1	0.0300
	MO	13	34.5	1119.3	0.0308
30+0	U+	3	2.5	37.7	0.0664
	M+	7	14	257.2	0.0472
30+30	MO	8	79.5	1876.4	0.0424
	U+	5	6	848.7	0.0071
	M+	9	38	654.5	0.0581
60+0	UO	4	40.5	2735.2	0.0148
	MO	8	44.5	1307.7	0.0340
60+0	U+	5	29.5	460.0	0.0641
	M+	6	83	629.9	0.1137
60+6h	UO	3	14.5	1719.7	0.0084
	MO	6	16.5	739.7	0.0223
60+6h	U+	4	11.5	289.3	0.0398
	M+	8	103	578.1	0.1782
15+0*	MO	10	77	841.2	0.0915
**	MO				0.2700
15+0*	M+	7	83.3	363.0	0.2300
**	M+				0.6900

MYOFIBRILS

12 weeks exposure. (* is 4 weeks, ** is corrected to 12 weeks).

EXPERIMENT	CELL	PLATES	GRAINS	AREA	SPEC.AC.
10+0	+	5	8.5	126.0	0.0675
10+30	+	5	79	15.8	0.0798
30+0	0	13	65.5	900.2	0.0728
	+	8	56	1525.0	0.0367
30+30	0	8	89.5	870.8	0.1028
	+	8	25	271.8	0.0920
60+0	0	8	15.5	251.7	0.0616
	+	6	101	514.0	0.1965
60+6 h	0	5	66.5	499.7	0.1331
	+	7	35.5	348.0	0.0892
15+0	0*	10	177	1242.6	0.1424
	0**				0.4200
15+0	+*	7	98.5	424.9	0.2318
	+**				0.6900

PARS FIBROSA

12 weeks exposure

EXPERIMENT	CELL	PLATES	GRAINS	AREA	SPEC AC.
10+0	U	3	0.5	12.9	0.0387
	M	4	5	7.5	0.6645
10+30	U	2	10	6.7	1.4825
	M	1	4	2.8	1.4100
30+0	U	1	0	1.7	0
	M	2	4	11.5	0.3470
30+30	U	2	0.5	129.8	0.0039
	M	1	3	2.0	1.5306
60+0	U	6	46.7	16.9	2.7663
	M	1	14.8	6.5	2.2699
60+6h	U	4	14	12.5	1.1245
	M	2	10	9.2	1.0599
15+0*	M	3	14.7	36.2	0.4082
**	M				1.2300

PARS GRANULOSA

EXPERIMENT	CELL	PLATES	GRAINS	AREA	SPEC.Ac.
10+0	U	3	2	25.9	0.0773
	M	4	7	17.6	0.3989
10+30	U	2	6	7.8	0.7646
	M	2	9.5	14.9	0.6357
30+0	U	1	1	3.1	0.3185
	M	2	2	8.3	0.2403
30+30	U	2	3.5	252.3	0.0139
	M	1	1.5	4.5	0.3341
60+0	U	7	28	17.4	1.6182
	M	1	33.8	15.5	2.1849
60+6h	U	4	11	17.3	0.6350
	M	2	23	24.0	0.9489
15+0	M*	3	18.3	47.7	0.3840
	**				1.1400

*4 weeks exposure ** corrected to 12 weeks exposure.

BLOBS

18 weeks exposure (* is 12 weeks, ** is corrected to 18 weeks).

EXPT.	CELL	GRAINS	AREA	SP.AC.	No OBSERVED	No LABELLED	% LABELLED
30+0*	U	0	1.8	0	11	0	0
*	M	2	13.4	0.15	30	2	7
**	U			0			
**	M			0.22			
30+30	M	12	18.7	0.64	12	4	33
60+0	U	5	5.4	0.92	5	4	80
	M	8	14.0	0.57	20	7	35
60+6h	U	3	35.9	0.08	23	3	13

BACKGROUND

12 weeks exposure. Area observed in each case = $14512\mu^2$.

EXPERIMENT	GRAINS	SPEC.AC.
WT60+0	0	0
WT60+6h	2	0.000138
WT30+30	2	0.000138
060+6h	0	0
WT30+0	2	0.000138
030+30	1	0.000069
010+0	1	0.000069
Mean	1.14	0.000089

(Σ = summation of squares of number of grains minus mean No. of grains)

SARCOMERE PROFILE DATA

12 and 18 week exposures pooled. X^2 is for 4 degrees of freedom

SARCO. POSN		0-10	11-20	21-30	31-40	41-50	TOTAL	MEAN	Σ	X^2	P
GRAINS	WT	5	9	10	6	6	36	7.2	18.8	2.6	0.5 - 0.7
%TOTAL	10+0	14	25	28	17	17	101				
GRAINS	WT	7	11	8	10	9	45	9.0	10	1.1	0.8 - 0.9
%TOTAL	10+30	16	24	18	22	20	100				
GRAINS	WT	10	7	6	5	6	34	6.8	14.8	1.22	0.8 - 0.9
%TOTAL	30+0	29	21	18	15	18	101				
GRAINS	WT	5	8	6	8	6	33	6.6	7.2	1.09	0.95-0.98
%TOTAL	30+30	15	24	18	24	18	99				
GRAINS	0-nu	11	10	6	11	5	43	8.6	33.2	3.86	0.3 - 0.5
%TOTAL	30+0	26	23	14	26	12	101				
GRAINS	0-nu	18	15	10	21	9	73	14.6	105.2	7.21	0.1 - 0.2
%TOTAL	30+30	25	21	14	29	12	101				
GRAINS	WT	10	8	9	13	11	51	10.2	16.9	1.66	0.7 - 0.8
%TOTAL	60+0	20	16	18	25	22	101				
GRAINS	WT	19	16	13	8	14	70	16	86	5.38	0.2 - 0.3
%TOTAL	60+6h	27	23	19	11	20	100				
GRAINS	0-nu	6	4	15	12	10	47	9.4	79	8.43	0.05-0.10
%TOTAL	60%0	13	9	32	26	21	101				
GRAINS	0-nu	7	4	6	12	10	39	7.8	40	5.23	0.2 - 0.3
%TOTAL	60+6h	18	10	15	31	26	100				

APPENDIX 7.4

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Abbreviations of journal names:

Arch. Bioch. Bioph.	Archives of Biochemistry and Biophysics.
BBA	Biochemica Biophysica Acta.
BBRC	Biochemical & Biophysical Research Communications
CSHSQB	Symposia on Quantitative Biology, Cold Spring Harbor Laboratory.
Current Topics in DB	Current Topics in Developmental Biology
DB	Developmental Biology
ECR	Experimental Cell Research
JBC	Journal of Biological Chemistry
JCB	Journal of Cell Biology
J.Cell Sci.	Journal of Cell Science
J.Histo.Cyto.	Journal of histochemistry and cytochemistry
JMB	Journal of Molecular Biology
J.Micros.	Journal de Microscopie
J.Ult.Res.	Journal of Ultrastructure Research

NCI Monog.	National Cancer Institute Monograph.
NNB	Nature New Biology
PNAS	Proceedings of the National Academy of Sciences of the United States.

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